On and after 1 January 1945, all communications should be addressed to Professor F. G. Young, St Thomas's Hospital Medical School, London, S.E. 1, and NOT at Godalming.

APR 7 1945

THIS PART COMPLETES VOLUME 38 (1944)

1944

VOLUME 38 No. 5

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

F. DICKENS
E. J. KING
B. C. J. G. KNIGHT
J. R. P. O'BRIEN
A. R. TODD
F. G. YOUNG (*Chairman*)

CAMBRIDGE UNIVERSITY PRESS
ENGLAND

Price 15*s. net*

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. Under present conditions the parts of each volume will not necessarily appear at regular intervals but will be issued as frequently as possible. During 1944 it is hoped that five parts will be published.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Hon. Secs., Dr W. T. J. Morgan, Lister Institute, Chelsea Bridge Road, London, S.W.1, and Dr W. Robson, Dept. of Physiology, King's College, Strand, London, W.C.2, or from the Hon. Treas., Dr J. H. Bushill, The Laboratories, Cadby Hall, London, W.14.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £3. 10*s*. 0*d*. per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1, to Dr J. H. Bushill, The Laboratories, Cadby Hall, London, W.14, or to any bookseller. In the United States of America the *Journal* may be purchased from the University of Chicago Press, 5750 Ellis Avenue, Chicago, Ill., the American agents of the Cambridge University Press for the sale of the *Biochemical Journal*.

Communications respecting the sale of single issues or back numbers of the *Journal* should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

In paper covers

Volumes I–VI.	31*s*. 6*d*. net per vol.
Volumes VII–X.	(*Out of print*.)
Index of Authors and Subjects in Volumes I–X.	5*s*. 3*d*. net. (By post, 5*s*. 5*d*.)
Volumes XI–XVI.	(*Out of print*.)
Volumes XVII–XVIII.	60*s*. net per vol.
Volume XIX.	(*Out of print*.)
Volumes XX–XXV.	60*s*. net per vol.
Volumes XXVI–XXXVI.	70*s*. net per vol.
Index of Authors and Subjects in Volumes XI–XX.	10*s*. net. (By post, 10*s*. 4*d*.)

Binding. Quotations can be given by the publishers for bound copies of back numbers; also for buckram binding cases, and for binding subscribers' sets.

Cereal Phosphatases

1. THE ASSAY OF FREE WHEAT PHOSPHOMONOESTERASE AND CHARACTERIZATION OF THE FREE PHOSPHATASES OF WHEAT

By R. G. BOOTH, *Cereals Research Station, Ministry of Food, St Albans*

(*Received* 27 *June* 1944)

Relatively little attention has been paid to plant phosphatases in comparison with those found in animal tissues, despite the fact that the first dephosphorylating enzyme discovered was phytase in cereal grains (Suzuki, Yoshimura & Takaishi, 1907). Phosphatases of various types have since been found to have a wide distribution, and it is increasingly clear that, together with the phosphorylases, they have an important role in carbohydrate and mineral metabolism in both plants and animals. The present study was undertaken following the observation that wheaten flour contains a phosphatase which, under appropriate conditions, will dephosphorylate co-carboxylase (aneurin pyrophosphate) more rapidly and efficiently than the phosphatase contained in taka-diastase.

Collatz & Bailey (1921) and Herd (1931) studied phosphatase activity of flours by measuring the electrical conductivities of aqueous flour extracts before and after incubation, but results were equivocal and at best this method is very non-specific. Berliner & Rüter (1928) provided evidence of a phosphatase of special type in wheat-flour extracts, which would dephosphorylate soya-bean phosphatide.

Uzawa (1932) reported that rice bran contained a phosphomonoesterase with pH activity optimum of *c.* 5·0. This enzyme was similar to several acid phosphatases of mammalian viscera in respect of optimal pH, relative rate of hydrolysis of α- and β-glycerophosphate ($\beta > \alpha$), and also in the absence of any activation by Mg^{++}. He also reported a phosphodiesterase associated with the monoesterase of rice bran, but, while he was able satisfactorily to free the monoesterase from diesterase, he was unable to free the diesterase from monoesterase.

Ignatieff & Wasteneys (1936) studied the distribution of phosphomonoesterase in wheat prior to and during germination, with β-glycerophosphate as substrate, while similar studies were made by Hilbe & Marron (1940) with the histological technique of Gomori (1939), in which sections of the material containing the enzyme were caused to produce calcium phosphate (and subsequently a grey-black stain from reduced silver phosphate). Nemec (1928) and Lüers & Malsch (1929) carried out similar studies, particularly on barley, also assessing phosphomonoesterase activity.

Sarma & Giri (1942) have demonstrated that the phosphomonoesterase of rice and ragi can exist in two forms, one 'bound' and not extractable from the finely powdered material by water, while the other portion is freely water-soluble. The marked increase in phosphomonoesterase activity noted on germination is only due in part to the concomitant freeing of 'bound' enzyme, and, therefore, a real increase occurs in the dephosphorylating activity of the grain as a whole as its metabolic activity becomes accelerated.

Giri (1941) has also shown that rice, barley, maize and other cereals (but not wheat) contain a pyrophosphatase. It has been established from the work of Takahashi (1932), Munemura (1933), Bauer (1935, 1936) and Giri (1936), by means of differential heat and pH inactivation and by adsorption techniques, that this enzyme is different from the phosphomonoesterase with which it is associated. This finding is supported by theoretical considerations of enzyme specificity.

In the enzymic hydrolysis of co-carboxylase by wheat extracts (which, as will be shown later, also contain pyrophosphatase), phosphomonoesterase is particularly concerned owing to the fact that acid solutions of co-carboxylase are unstable and spontaneously become converted to aneurin monophosphate + phosphoric acid.

The excellent review of phosphatases by Folley & Kay (1936) includes a systematic classification in which wheat phosphatases do not appear. It is desirable to know whether such cereal phosphatases are at all typical in respect of any of the characteristics selected in order to effect this classification.

The study of cereal phosphatases is beset by some primary problems which have so far been only partially surmounted. As already mentioned, Sarma & Giri (1942) have shown that the enzymes occur in 'free' and 'bound' states, and that aqueous extraction of grain removes only the 'free' phosphatase. A point of major importance with cereals is that in the normal grain or flour there is too little moisture

present for much enzyme activity to occur, e.g. respiration rate in normal grain is very slow. Immediately the grain is moistened or the flour is taken up in water, enzyme-substrate reactions occur more readily and orthophosphate is liberated from compounds of phosphorus in the grain, a certain portion of the phosphatase being concomitantly destroyed. This effect is lowered by reducing the temperature at which extraction is carried out, but this brings about a reduced rate of extraction of the enzymes.

Preliminary experiments, however, indicated that so far as phosphomonoesterase activity is concerned, the gain in rapidity of extraction at 20° as compared with 4° much more than offsets the small loss of activity due to increased enzyme action at the higher temperature. All extractions were therefore carried out at room temperature (approx. 20°). The following work is concerned almost exclusively with 'free' cereal phosphatase as found in raw non-germinated grain.

METHODS

Rapid methods of assay of cereal phosphomonoesterase activity in terms of already established units

Probably the most widely used method for the estimation of phosphatase activity (in blood serum, plasma, etc.) is that of King & Armstrong (1934), which employs disodium monophenylphosphate as substrate. The method is designed to measure mammalian alkaline phosphomonoesterase and achieves considerably greater sensitivity and speed than methods using β-glycerophosphate as substrate, such as that of Bodansky (1932, 1933). It appeared to be very convenient for use with wheat extracts because such extracts contain no phenol, and the amount of inorganic phosphorus in them is too small to affect appreciably the velocity of the reaction employed.

King & Armstrong's method has been modified by Gutman & Gutman (1938*a*, *b*, 1940) for the assay of mammalian acid phosphatase by changing the buffer from veronal to citrate of pH 4·9. For assay of cereal phosphatase, many of the considerations which led to the choice of citrate buffer and a pH of 4·9 do not obtain, e.g. there is no alkaline phosphatase present in wheat extracts (see later), and therefore there is no reason why buffering should not take place at the enzyme optimum; there is further no reason why citrate (with its slightly inhibitory effect upon final molybdate colour development) should be used in preference to acetate buffer.

The pH optimum of wheat phosphomonoesterase is 5·15 (Fig. 2) and, in the preparation of buffer substrate for this pH, cognizance was taken of the fact that the addition of disodium monophenylphosphate causes a slight alkaline shift in acetate buffer. The only fundamental difference in the following methods A and B lies in the phenol reagents used.

Phosphatase assay—method A: modification of the procedure of King & Armstrong (1934)

Reagents

Buffer substrate consisting of M/200 (1·09 g./l.) of ether-washed, phenol-free disodium phenylphosphate dissolved in M/5 sodium acetate-acetic acid buffer of pH 5·1. This reagent is prepared weekly from a stock solution of buffer, and, after saturation with chloroform, is preserved in the refrigerator. The solid disodium phenylphosphate is also kept at 4°.

Folin & Ciocalteu (1929) *reagent* diluted 1 in 3 with distilled water.

Na_2CO_3 *solution*, 20% (w/v) of anhydrous Analar reagent in distilled water.

Procedure

Two 4 ml. samples of buffer substrate are pipetted into two tubes in a water-bath at 38°, and to one tube (the control) is added 1·8 ml. of diluted Folin & Ciocalteu reagent. After temperature equilibrium has been established, 0·2 ml. of a previously centrifuged and filtered flour extract is added to both tubes. The contents of the tubes are well mixed, and precisely 30 min. after the addition of the flour extract to the second tube the Folin & Ciocalteu reagent is added to this tube also. Unlike the original King & Armstrong method, no precipitate forms at this stage, owing to the exceedingly small amount of protein present in the flour extract. Filtering is therefore unnecessary, and to each of the tubes is added 1·5 ml. of 20% (w/v) Na_2CO_3 solution; they are mixed with a footed glass rod and allowed to remain in the bath for 10 min. for full colour development. Comparison is made in the Spekker photoelectric absorptiometer using 1 cm. cells and Ilford Spectrum Red (no. 608) filters, the instrument having been previously calibrated against standard phenol solutions over a suitable range.

Phosphatase assay—method B: modification of the Scharer (1938) *method*

Reagents

Buffer substrate as already described for method A.

Alkaline buffer solution made by mixing equal volumes of M/5 boric acid in M/5 KCl, and N/2 × 1·07 NaOH solution.

Solution of 2:6-*dibromoquinone-chloroimide*, 0·04 g. in 10 ml. of 95% ethanol, prepared weekly and kept in an amber glass bottle. A suitable dropper giving 0·18–0·22 mg. of chloroimide/3 drops should be chosen for use with the reagent.

Procedure

Two 5 ml. samples of buffer substrate are pipetted into two tubes in the water-bath at 38°. To the control tube is added 2 ml. of alkaline buffer solution (borate-NaOH) followed by 0·2 ml. of flour extract. After temperature equilibrium has been established, 0·2 ml. of flour extract is added to the second tube. After precisely 30 min., 2 ml. of alkaline buffer solution are added to this tube and the contents are mixed. Both tubes are removed from the bath and allowed to cool and 3 drops of chloroimide reagent are added to each. The indophenol blue colour is allowed to develop for 15 min., after which the tubes are matched in the same way and with the same filters as in method A.

When using method B, it is necessary to ensure (1) that the pH of the resulting solution after the alkaline buffer is added is 9·5±0·2, as otherwise colour development is interfered with, and (2) that no saliva (containing alkaline salivary phosphatase) finds its way into the tubes. NaF may be added to the alkaline buffer solution at the rate of 5 g./l. to inhibit the action of any such alkaline phosphatase.

In the use of either of methods A or B, extreme cleanliness of tubes, flasks, etc., is essential to success and reproducibility of results, and chromic acid cleaning has been found to be the only safe way of avoiding trouble from this source.

Probable error of methods A and B

Method A has given a probable error on triplicate determinations of $\pm 2{\cdot}7\%$ ($P = 0{\cdot}99$), while the comparable figure for method B is $\pm 2{\cdot}95\%$. There is, therefore, little to choose between them on this score.

Expression of results

The unit of phosphatase as defined by King & Armstrong (1934) is the amount of enzyme which will set free 1 mg. of phenol under the conditions of the test. Instead of quoting units/100 ml. of serum, the content/g. is a convenient form of expressing the activity of cereals. Thus the free phosphatase activity of a cereal, in units, is the number of mg. of phenol freed by the aqueous extract from 1 g. of the cereal under the conditions of the test. The advantage of this means of expression lies principally in the fact that it avoids the setting up of new units and draws a close parallel between plant and animal assays.

Preparation of cereal extracts

Distilled water saturated with chloroform was used for extractions, and the relative amounts of flour and water used were kept constant at 1 g. + 25 ml. After being centrifuged and filtered, this extract was diluted 1 in 6 when normal whole-wheat flour extracts were being assayed, a greater dilution being necessary or germinated (or malted) cereals and a lesser dilution for low extraction flours or samples in which the enzyme has been inactivated by heat or other means.

EXPERIMENTS

The rate of extraction of phosphatase from finely ground whole-cereal grains was studied, using method A for assay. In Table 1 are given the phosphatase contents indicated after various periods of extraction (with frequent shaking). The flour residues after 24 hr. extraction were again extracted with a fresh lot of 25 ml. of water, and this second extraction was allowed to proceed for 12 hr. A third aqueous extraction failed to remove any measurable amount of phosphatase, but assays on the solid residue showed high 'bound' activity.

Table 1. *Free phosphatase content of whole-grain flour as indicated after various lengths of aqueous extraction*

	Time of extraction (King-Armstrong units)						
Cereal	½ hr.	1 hr.	2 hr.	4 hr.	8 hr.	24 hr.	2nd extraction (12 hr.)
Wheat	11·4	12·3	12·45	12·9	13·7	14·8	1·4
Oats	5·25	5·55	6·00	7·5	8·1	8·85	0·00
Barley	2·1	2·25	3·2	3·4	3·65	4·00	0·9

The rate of extraction of the enzyme is clearly so slow between 8 and 24 hr. that little inaccuracy is introduced by adopting a convenient time of 16–20 hr. as standard for the extractions; no significant differences occurred between assays carried out on extracts made within this time range.

It is worthy of note that the amount of enzyme extracted by water under standard conditions of time and temperature is not independent of the relative amounts of flour and water used. Thus, 1 g. of wholemeal flour extracted with 150 ml. of water gives an extract of consistently and markedly lower activity than that of the same flour extracted by the standard method described above (1 g. + 25 ml. extract diluted 1 in 6 after centrifuging).

The determination of various characteristics of wheat phosphomonoesterase

Determination of optimal pH *of wheat phosphomonoesterase*

This determination was carried out on four different substrates: aneurin monophosphate (acidified solution of co-carboxylase), disodium phenylphosphate and α- and β-glycerophosphates, with acetate buffer (and in one instance phthalate also). As will appear later, there are probably at least two phosphomonoesterases in wheat, and the optimal pH found here refers therefore to the presumptive mixture of them as naturally occurring. As no success has yet attended efforts to separate entirely the two (or more) enzymes, their individual pH optima, if different, cannot yet be determined, but measurements on wheat enzyme fractions which appeared to contain markedly different proportions of the two enzymes have shown no significant departure from the values reported here on the naturally occurring mixture.

Preliminary experiments indicated an optimal pH of approximately 5·0. The following are the protocols of a typical experiment on the more accurate determination of this pH.

Acetate buffer of strength M/10 was made up at nominal pH's from 4·75 to 5·25 at steps of 0·05 pH. 5 g. of whole-ground wheat were suspended in 95 ml. of buffer (using a Waring blendor), a few drops of toluene were added, and extraction was allowed to proceed at room temperature for 16 hr., with frequent agitation. The flour was centrifuged off, the extract was made up to 95 ml. and its pH was determined (to 0·01 pH) electrometrically with a glass electrode. It was then placed in a flask and immersed in a water-bath at 38° until equilibrium was established. 5 ml. of a previously acidified solution of Merck co-carboxylase (54·6 mg./l.) in the appropriate buffer at 38° were added rapidly, the solutions were mixed, the time was noted, and samples were withdrawn at 5, 10, 30 min., 1 and 2 hr. intervals. Immediately they were withdrawn, these samples were run into an equal volume of ice-cold M/20 NaF solution which, as had been previously shown, effectively inhibited further enzyme activity. Aneurin was measured in these samples immediately by the photoelectric fluorimetric technique evolved by the present author and described in a report of the Accessory Food Factors Committee of the

Medical Research Council (1943). The velocity of the enzymic dephosphorylation of the aneurin monophosphate is almost linear for the first 15 min. under these conditions, despite the low substrate concentration, as can be seen from Fig. 1.

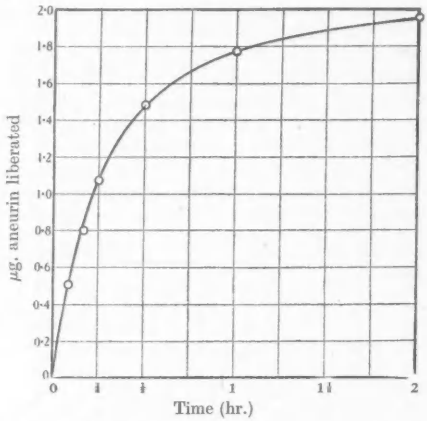


Fig. 1. The dephosphorylation of aneurin monophosphate by aqueous flour extract in M/10 acetate buffer at pH 5·0; initial substrate concentration 2·73 μg. of co-carboxylase/ml.

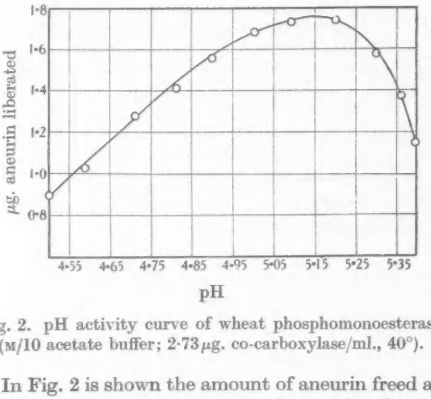


Fig. 2. pH activity curve of wheat phosphomonoesterase (M/10 acetate buffer; 2·73 μg. co-carboxylase/ml., 40°).

In Fig. 2 is shown the amount of aneurin freed at various pH's after a constant time of incubation (½ hr.), from which it is clear that the optimum pH of the enzyme lies between pH 5·1 and 5·2. Experiments similar to the above, using M/200 α- and β-glycerophosphate as substrates and using Allen's (1940) method for estimation of freed inorganic phosphorus, and experiments using the method outlined for assay of phosphatase activity in cereal extracts with phenylphosphate and buffers of varying pH, have confirmed the optimal pH at 5·15. The activity is still very considerable at pH 4·0 but disappears between 4·0 and 3·0; on the alkaline side activity disappears between pH 6·0 and 7·0. By reason of these facts and that co-carboxylase and aneurin do not lose vitamin activity in solution at pH 4·0 and 38° overnight, a wheat extract (dialyzed free of aneurin) is a useful source of phosphatase in the assay of co-carboxylase by chemical means and has been found to be superior to taka-diastase in many respects. This subject will be dealt with more fully elsewhere.

Experiments on precisely similar lines to those described above, but using various dilutions of enzyme solution with a constant amount of buffer substrate of fixed concentration, have shown that the velocity of the dephosphorylation reaction is directly proportional to enzyme concentration over a wide range.

Wheat contains no alkaline phosphomonoesterase, this having been demonstrated on a disodium phenylphosphate substrate at various pH's ranging from 8 to 10, with borate, veronal and glyoxaline buffers. This observation accords well with the fact that the pH of wheat, either resting or during germination, lies below 6·0.

The thermal destruction of free wheat phosphomonoesterase in solution and its critical inactivation temperature

Preliminary experiments carried out on dialyzed wheat extracts buffered with acetate at pH 5·15 and in the absence of added substrate indicated that destruction was at an appreciable rate at 45° and became very rapid at over 60°. More detailed experiments were carried out on 24 hr., room temperature, 20% extracts of finely ground wholewheat flour in chloroform water, which were centrifuged and filtered and then dialyzed in cellophan (sausage skin) bags for 12 hr. against flowing tap water, then for a further 36 hr. against frequently changed distilled water.

Ninety ml. of 0·25 M-sodium acetate-acetic acid buffer, pH 5·15, in a 150 ml. Erlenmeyer flask, were immersed in a water-bath maintained at the desired temperature ±0·25° and were allowed to reach temperature equilibrium. 10 ml. of the dialyzed enzyme solution were then run into the flask, which was agitated until temperature equilibrium was reached again (1½–2 min.), the time of addition having been noted on a stop-watch. At precise time intervals (noted in Fig. 3) small samples were withdrawn and run into thin-walled ½ in. test-tubes immersed in an ice-bath. Phosphatase assays were carried out on these samples immediately, and after 24 and 48 hr. in the refrigerator, by the modified King-Armstrong method (A) previously described. The destruction observed under these conditions appears to be irreversible and is constant for any given temperature/time over wide ranges of enzyme and buffer concentrations. It therefore appears to be true thermal destruction. Fig. 3 indicates some of these rates of destruction graphically. The critical inactivation temperature (i.e. that temperature at which 50% of the enzyme activity is

destroyed in 1 hr.) is 53°. It is of interest to note that the critical inactivation temperature of wheat protease as determined by Mounfield (1936) is 54°.

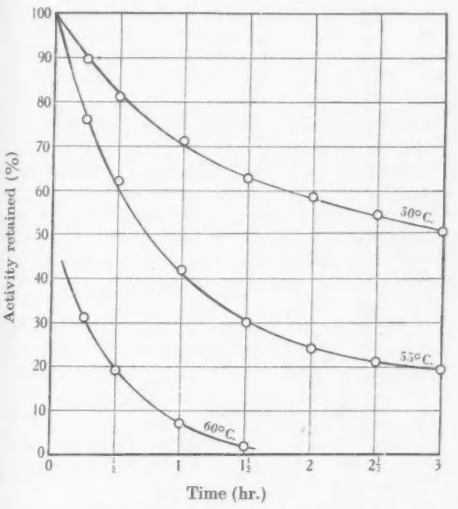


Fig. 3. The thermal destruction of free wheat phosphomonoesterase in M/20 pH 5·15 acetate buffer.

Prior to extraction from the wheat grain, the phosphomonoesterase is very much more resistant to heat treatment. For example, it has been shown that no loss of enzyme occurs in wheat of 14% moisture content after heating at 67° for 36 min., and if wheat of very low moisture content be used this temperature may be raised to 90–100° without appreciable loss. As might be expected from theoretical considerations, this destruction of free phosphatase *in situ* is closely bound up with protein denaturation. This subject, with its many practical implications, will be dealt with elsewhere.

Activation and inhibition of free wheat phosphomonoesterase

Wheat-enzyme preparations were made by ethanolic precipitation of centrifuged and filtered 20% aqueous extracts of wheat. The precipitates were taken up in water, filtered and dialyzed against frequent changes of distilled water for 48 hr. They were then found to be free of Mg^{++} when tested by the method of Weisselberg (1931) with *p*-nitrobenzene-azo-α-naphthol. The volumes of the solutions were adjusted to give suitable activities for use on disodium phenylphosphate and β-glycerophosphate (M/200) substrates at pH 5·15.

The results obtained with various substances which commonly act as activators and inhibitors of phosphatases are shown in Table 2. The absence of

Table 2. *Activation and inhibition of free wheat phosphomonoesterase* (M/200 *substrate in* M/20 *buffer*, pH 5·15)

Substance	Concentrations used	Effect
Mg^{++} (as $MgSO_4$)	$M \times 10^{-6}$ to 10^{-4}	None
HCN	$M \times 10^{-2\cdot7}$ to 10^{-5}	None
Iodoacetic acid	$M \times 10^{-2}$ to 10^{-6}	None
NaF	$<M \times 10^{-4\cdot7}$	None
NaF	$>M \times 10^{-4\cdot7}$	Some inhibition
NaF	$>M \times 10^{-2}$	Total inhibition

Mg^{++} activation is in accord with the findings noted in the review of Folley & Kay (1936) in respect of rice bran phosphomonoesterase and the mammalian acid phosphoesterases with which the rice-bran enzyme is classed.

The nature of fluoride inhibition of wheat phosphomonoesterase

Disodium phenylphosphate buffer substrate of pH 5·0 (M/20 acetate buffer) was prepared at M/200, M/100 and M/50 strengths, each solution being also made $M \times 10^{-3}$ in respect of NaF. This concentration of NaF had been previously shown to bring about approximately 60% inhibition of activity in M/200 substrate. These solutions were treated in precisely the same manner as in determining the unit activity of cereal extracts, using a purified enzyme solution in suitable quantity, the same amount of enzyme solution being added to each tube. Similar experiments were carried out in the absence of fluoride.

The percentage inhibition of the constant quantity of enzyme used in each case was also roughly constant, indicating therefore that NaF is non-competitive but exerts its effect directly as a 'poison' to the enzyme.

The various free phosphatases of wheat

(*a*) *Phosphomonoesterases.* In the experiments on determination of optimal pH it was noticed that the activity of a given wheat-enzyme solution appeared to be greater in respect of α- than β-glycerophosphate despite the pH optima for the two substrates being identical. This observation was not in accord with the findings of Uzawa (1932) with rice-bran phosphomonoesterase, which dephosphorylated β- very much more rapidly than α-glycerophosphate.

In Fig. 4 is illustrated a typical experiment on a wheat extract. The freed orthophosphate was estimated by the method of Allen (1940) modified by the replacement of the 60% perchloric acid by 5N-H_2SO_4. (Pringle (1943) has shown that the replacement of 60% perchloric acid by 5N-H_2SO_4 (after the work of Berenblum & Chain, 1938) is quite satisfactory.) This 5N-H_2SO_4 was used to stop the enzymic reaction after the appropriate time intervals.

It is clear that α- is hydrolyzed much more rapidly than β-glycerophosphate by aqueous extracts of wheat. It was therefore thought possible that wheat might contain two phosphomonoesterases, one similar to that described by Uzawa (hydrolyzing β- more rapidly than α-glycerophosphate) and another

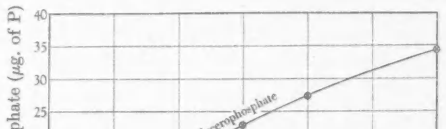


Fig. 4. The dephosphorylation of α- and β-glycerophosphate (0·004 M in M/20 acetate buffer pH 5·1) at 37° by centrifuged and filtered aqueous extracts of wheat. 4 ml. of substrate + 4 ml. of wheat extract used in each case.

present to a greater extent and showing an opposite relative affinity for the two glycerophosphate substrates. To test this theory, aqueous extracts of wheat were fractionated as follows:

(i) by precipitation with $(NH_4)_2SO_4$ followed by dialysis of the re-dissolved precipitate,

(ii) by precipitation with ethanol followed by dialysis of the re-dissolved precipitate.

In the case of (i) precipitation was carried out in three stages: (*a*) $\frac{1}{3}$ saturation, (*b*) $\frac{1}{3}$ to $\frac{2}{3}$ saturation, and (*c*) $\frac{2}{3}$ to full saturation of the initial filtered 20% aqueous wheat extract. Fraction (*b*) was most active, (*c*) almost inactive, and (*a*) possessed an activity about one-sixth that of (*b*), but all fractions behaved in the same way in respect of more rapid hydrolysis of α- than β-glycerophosphate. The behaviour of 50 and 50–75% ethanol precipitation fractions was similar to that obtained with the ammonium sulphate. Pairs of enzyme solutions were prepared from fractions (*a*) and (*b*) and also from the ethanol precipitations which were of equal activity in respect of disodium phenylphosphate, and these were used in suitable equal amounts on α- and β-glycerophosphate substrates. The results obtained in respect of ethanol fractions are shown in Figs. 5 *a*, *b*.

It is clear that the ratios of rate of hydrolysis of α-:β-glycerophosphate vary markedly from fraction to fraction. This circumstance is difficult to explain except on the assumption that wheat extracts contain two or more phosphomonoesterases which differ in their relative affinities for the two glycerophosphates.

(*b*) *Free phosphatases other than phosphomonoesterases in wheat.* The fractionated enzyme solutions used in the immediately preceding experiments were applied to other substrates, namely, diphenyl- and triphenylphosphates, sodium pyrophosphate and hexosediphosphate, all at M/200 in chloroform-saturated M/20 acetate buffer, pH 5·0. 4 ml.

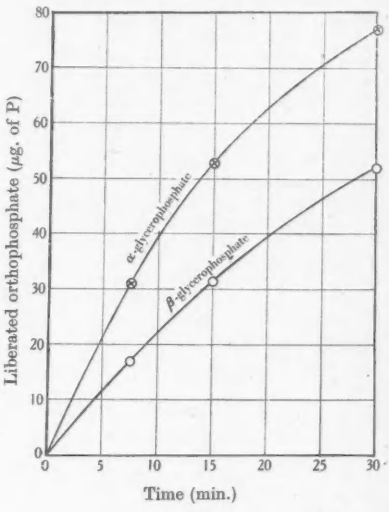


Fig. 5*a*.

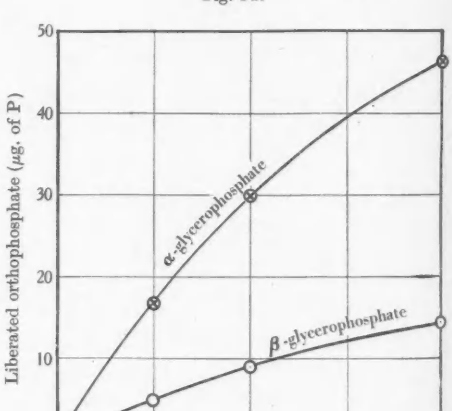


Fig. 5*b*.

Fig. 5. The dephosphorylation of α- and β-glycerophosphate (0·004 M in M/20 acetate buffer) by (*a*) '50% ethanol' precipitate from aqueous wheat extract; (*b*) '50–75% ethanol' precipitate from aqueous wheat extract.

samples of both buffer substrate and enzyme solutions were used as in the previous experiments, the reactions being stopped in the case of the phenylphosphates by the Folin & Ciocalteu reagent (prior to the estimation of free phenol)

and with 2 ml. of 5 N-H_2SO_4 (prior to the estimation of orthophosphate by Allen's method) in the case of the pyrophosphate and hexosediphosphate. Control tubes were incubated (with the enzyme reaction stopped by the appropriate reagent) for the same length of time and under precisely the same conditions as the experimental tubes.

In contrast with monophenyl-, triphenylphosphate was completely unaffected by the wheat-enzyme preparations and diphenylphosphate was very much less readily attacked. In the latter case, the $\frac{1}{3}$ saturation fraction had about $\frac{1}{50}$ of the activity on diphenyl- that it exhibited on monophenylphosphate, whereas the $\frac{1}{3}$ to $\frac{2}{3}$ saturation fraction has a comparable figure of only about $\frac{1}{250}$. This finding takes no account of the fact that the phosphodiesterase involved may have a different pH optimum from the phosphomonoesterases previously dealt with, but raises the possibility that the phosphodiesterase is either (1) heterogeneous or (2) has a solubility in ammonium sulphate solutions which differs markedly from the solubilities of the phosphomonoesterases.

The test as carried out here is undoubtedly a measure of diesterase activity at pH 5·0, as, owing to the very much greater speed of the monoester reaction, the overall speed of the reaction is entirely governed by the activity of the diesterase.

Sodium pyrophosphate and hexosediphosphate were hydrolyzed by both wheat-enzyme fractions, solutions of equal activity in respect of monophenylphosphate substrate showing in both instances almost double the activity in the $\frac{1}{3}$ to $\frac{2}{3}$ saturation fraction of that in the $\frac{1}{3}$ saturation fraction. This $\frac{1}{3}$:$\frac{1}{3}$ to $\frac{2}{3}$ ratio of about 1 : 2 here is in marked contrast to the comparable ratio of approximately 5 : 1 in respect of diesterase activity.

It does, however, strengthen the probability that both the pyrophosphatase and hexosediphosphatase of wheat are not heterogeneous.

DISCUSSION

The concept of 'bound' and 'free' phosphatase in cereals put forward by Sarma & Giri (1942) has been confirmed, but it is clear that the proportions of 'bound' and 'free' enzyme are not absolute in the way that, for example, solubilities of salts are absolute. The way in which the amount of 'free' phosphatase varies with differing relative amounts of solid and liquid phase during extraction suggests that the enzyme is behaving in a manner similar to that found with proteins—within limits, the higher the concentration of salts (extracted from the flour), the higher the solubility. Preliminary experiments on the extraction of phosphatase with protein-dispersing solvents (2 % aqueous KCl, 3 % aqueous KBr, etc.) tend to confirm the view that the 'free' phosphatase is probably bound to, or closely associated with, the soluble proteins of wheat in the same way that adenosinetriphosphatase is associated with myosin. The finding of Sarma & Giri that some of the 'bound' phosphatase of rice and ragi becomes freed on germination also supports this view, as the proteolysis which takes place on germination would tend to free the phosphatase previously bound to the insoluble proteins of the grain.

The high concentration and variety of phosphatases contained in wheat clearly signify that their role in germination and the utilization of the food reserves in the endosperm of the grain must be a major one. Ignatieff & Wasteneys (1936) observed that the concentration of phosphatase in the leaves is higher than that in any other part of the plant during its entire life period, which would indicate that the enzyme plays a part in the intense metabolic activity which takes place in such tissue—perhaps a part in photosynthesis. It would seem, therefore, that plant phosphatases must play quite as vital a part in plant metabolism as they do in animals.

SUMMARY

1. Two rapid methods are described for the assay of free phosphomonoesterase activity in wheat and other cereals in terms of already established units.

2. The optimal pH of free wheat phosphomonoesterase was found to be 5·15. The activity is still considerable at pH 4·0, but disappears between pH 4·0 and 3·0 on the acid side and between 6·0 and 7·0 on the alkaline.

3. The rate of thermal destruction of this phosphatase in solution has been determined; it is appreciable at 45° and very rapid at 60° and over. The critical inactivation temperature is 53° and this thermal destruction is irreversible.

4. Neither $MgSO_4$ nor HCN activated wheat phosphomonoesterase, the former at 10^{-6} to 10^{-4} M and the latter at $10^{-2\cdot7}$ to 10^{-5} M concentrations. Iodoacetic acid did not inhibit the enzyme at 10^{-2} to 10^{-6} M, but NaF was strongly inhibitory.

5. Evidence is presented to show that there are probably at least two phosphomonoesterases in wheat which differ in their relative solubilities in ethanol and $(NH_4)_2SO_4$ solutions, and of which one at least dephosphorylates α-glycerophosphate more rapidly than β-glycerophosphate. The total free phosphomonoesterase activity of wheat is greater in respect of α- than β-glycerophosphate.

6. Wheat also contains free hexosediphosphatase and pyrophosphatase (probably single enzymes in each case) and a phosphodiesterase which may possibly be heterogeneous.

The author's thanks are due to Dr W. T. J. Morgan for a sample of pure hexosediphosphate and to Messrs Albright and Wilson for preparing and supplying a sample of pure monosodium diphenylorthophosphate.

REFERENCES

Accessory Food Factors Committee of the Medical Research Council (1943). *Biochem. J.* **37**, 433.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bauer, E. (1935). *Naturwissenschaften*, **51**, 866.
—— (1936). *Hoppe-Seyl. Z.* **239**, 195.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Berliner, E. & Rüter, R. (1928). *Z. ges. Mühlenw.* **4**, 29.
Bodansky, A. (1932). *J. biol. Chem.* **99**, 197.
—— (1933). *J. biol. Chem.* **101**, 93.
Collatz, F. A. & Bailey, G. H. (1921). *Industr. Engng Chem.* **13**, 337.
Folin, O. & Ciocalteu, F. (1929). *J. biol. Chem.* **73**, 627.
Folley, S. J. & Kay, H. D. (1936). *Ergebn. Enzymforsch.* **5**, 189.
Giri, K. V. (1936). *Curr. Sci.* **4**, 833.
—— (1941). *Ann. Biochem. exp. Med.* **1**, 297.
Gomori, G. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 23.
Gutman, A. B. & Gutman, E. B. (1938*a*). *Proc. Soc. exp. Biol., N.Y.*, **38**, 470.
—— —— (1938*b*). *J. clin. Invest.* **17**, 473.
—— —— (1940). *J. biol. Chem.* **136**, 201.
Herd, C. W. (1931). *Cereal Chem.* **8**, 145.
Hilbe, J. J. & Marron, T. V. (1940). *Proc. Iowa Acad. Sci.* **47**, 235.
Ignatieff, V. & Wasteneys, H. (1936). *Biochem. J.* **30**, 1171.
King, E. J. & Armstrong, A. R. (1934). *Canad. med. Ass. J.* **31**, 376.
Lüers, H. & Malsch, L. (1929). *Wschr. Brau.* **46**, 143.
Mounfield, J. D. (1936). *Biochem. J.* **30**, 549.
Munemura, S. (1933). *J. Biochem., Tokyo*, **17**, 343.
Nemec, A. (1928). *Biochem. Z.* **202**, 229.
Pringle, W. J. S. (1943). Private communication.
Sarma, M. L. & Giri, K. V. (1942). *Ann. Biochem. exp. Med.* **2**, 17.
Scharer, H. (1938). *J. Dairy Sci.* **21**, 21.
Suzuki, V., Yoshimura, K. & Takaishi, M. V. (1907). *Tokyo Imp. Univ. Coll. Agric. Bull.* **1**, 503.
Takahashi, H. (1932). *J. Biochem., Tokyo*, **16**, 447.
Uzawa, T. (1932). *J. Biochem., Tokyo*, **15**, 1.
Weisselberg, F. (1931). Quoted by Feigl, F. (1937). *Qualitative Analysis by Spot Tests.* New York: Nordemann Publishing Co.

Studies on Diffusing Factors

4. THE ACTION OF REDUCING AGENTS ON HYALURONIC ACID AND OTHER POLYSACCHARIDES

By C. W. HALE, *The Lister Institute of Preventive Medicine, Elstree, Herts*

(*Received* 19 *February* 1944)

Robertson, Ropes & Bauer (1940) suggested that the spontaneous loss of viscosity of vitreous humour might be due to its high ascorbic acid content. McClean & Hale (1940) showed that ascorbic acid, with or without hydrogen peroxide, increased dermal permeability and lowered the viscosity of hyaluronic acid. Thiolacetic acid, quinol, pyrogallol, 'metol', sodium sulphite and hydrogen sulphide caused a fall in the viscosity of hyaluronic acid; this fall was not immediate but progressed over a period (McClean & Hale, 1941). Activation of an enzyme present in the hyaluronic acid solution was excluded by preheating the solution. No reducing sugar could be detected in the substrate after its viscosity had been lowered by reducing agents, as it can after hydrolysis by hyaluronidase.

Robertson, Ropes & Bauer (1941) showed that the viscosity of other polysaccharides, such as starch and pectin, also could be decreased by reducing agents; not all organic reducing agents were equally effective, and the presence of hydrogen peroxide appeared to be necessary, since the effect was inhibited by catalase. When their viscosity was decreased in this way the polysaccharides were no longer precipitable as asbestos-like clots. It was suggested that the change of viscosity might be due to a dephosphorylation of the viscous polysaccharide. Pirie (1942) showed that hyaluronic acid obtained from tumours contained copper, that the action of ascorbic acid on hyaluronic acid was catalyzed by copper, and that this could be inhibited by sodium diethyldithiocarbamate.

The present work was undertaken to investigate the action of reducing agents on hyaluronate and other polysaccharides and the part played by copper.

RESULTS

Sodium sulphate and oxidized thiolacetic acid or the oxidation products of ascorbic acid (all 0·01M-solutions) do not lower the viscosity of the hyaluronate (Table 1). Thus it may be presumed that the progressive lowering of viscosity referred to above depends upon the presence of an active reducing agent and not upon alterations in the salt content.

Table 1. *Influence of several solutions on the viscosity of hyaluronate*

Solution (1 vol. solution + 5 vol. 0·25% buffered hyaluronate)	Result after 1 hr.
0·01 M-Na_2SO_3	Fall in viscosity: 4·26–3·14
0·01 M-Na_2SO_4	No fall in viscosity
0·01 M-cysteine	Fall in viscosity: 4·26–2·93
Saturated *l*-cystine	No fall in viscosity
0·01 M-thiolacetate	Fall in viscosity: 3·2–2·44
0·01 M-thiolacetate oxidized in air until it would not reduce $FeCl_3$	No fall in viscosity
0·01 M-ascorbic acid	Fall in viscosity: 3·2–1·28
0·01 M-ascorbic acid oxidized in air until it would not reduce indophenol in 5% trichloroacetic acid	No fall in viscosity

The rate of degradation* is not influenced by the concentration of salts present which is known to affect the activity of hyaluronidase (McClean & Hale, 1941; Madinaveitia & Quibell, 1941; McClean, 1943).

Effect of ascorbic acid on the viscosity of starch, pig gastric 'mucin' and gelatin compared with that on hyaluronate. Confirming Robertson *et al.* (1941), it has been found that ascorbic acid and other reducing agents lower the viscosity and destroy the characteristic precipitability of other polysaccharides besides hyaluronic acid, e.g. starch paste and 'mucin' from pig gastric mucosa (Fig. 1). The viscosity of gelatin is not altered. It was found that the products after the degradation of hyaluronate or starch were not dialyzable through a cellophan membrane, and that after the action of reducing agents starch still gave an unmodified iodine reaction (Table 2). There was no evidence of liberation of *N*-acetylglucosamine from hyaluronate, nor was the degraded hyaluronate precipitated from aqueous solution by any concentration of ethanol or acetone up to 95% by volume. Enzymic hydrolysis of either starch or hyaluronate after degradation by ascorbic acid + Cu is slower than that of the unchanged polysaccharides. Seshagirirao & Giri (1942) reported the reduction in the rate of hydrolysis by amylolase and J. H. Humphrey has observed (personal communication) a similar result with hyaluronidase.

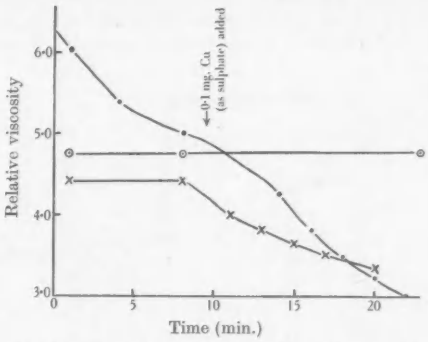


Fig. 1. Influence of ascorbic acid and Cu on the viscosity of starch, gelatin and 'mucin' from pig gastric mucosa. 2·5 ml. solution of polysaccharide or protein and 0·5 ml. ascorbic acid (final concentration 0·01 M), pH 7·0. •—• Potato starch 6 mg./35 ml. ⊙—⊙ Gelatin, 1%. ×—× 'Mucin' from pig gastric mucosa (Wilson, Type 1701-W) 0·5%.

Table 2. *Action of ascorbic acid on hyaluronate and potato starch*

(0·5 mg. Cu (as sulphate) and chloroform to saturate, added to each sample. Mixtures each contained in cellophan tube suspended in 20 ml. of water at 4° for 48 hr. Hydrolysis with 100 v.r. units (McClean, 1943) of *Cl. welchii* hyaluronidase for 6 hr. at 37°.)

Mixtures: Potato starch (6 mg./35 ml.) (ml.)	Mixtures: Hyaluronate (0·25%) (ml.)	Mixtures: Ascorbic acid	Results after dialysis	Viscosity	Action of 20 vol. ethanol or acetone	Iodine reaction	*N*-Acetyl glucosamine	Results after hydrolysis with *Cl. welchii* hyaluronidase. *N*-Acetyl glucosamine
10	—	M/600	Diffusate	—	—	Negative	—	—
			Dialysate	Decreased	No precipitate	Positive	—	—
—	10	M/600	Diffusate	—	—	—	Negative	Nil
			Dialysate	Decreased	No precipitate	—	Negative	Strong reaction
10	—	—	Diffusate	—	—	Negative	—	—
			Dialysate	Not decreased	Flocculent precipitate	Positive	—	—
—	10	—	Diffusate	—	—	—	Negative	Nil
			Dialysate	Not decreased	Mucin clot	—	Negative	Strong reaction

Influence of oxygen and of reduced oxidation-reduction (O/R) *dyes on hyaluronate.* The following

* In this paper the term 'degradation' means that the polysaccharides suffer an irreversible change from their naturally occurring physical condition and does not necessarily imply that any measurable chemical change has taken place. Evidence that the macromolecules of the viscous polysaccharides undergo disruption to smaller units is provided by a marked fall in viscosity and loss of precipitability from mixtures of organic solvents and water.

dyes in the reduced form (0·01 M, pH 7·0) did not lower the viscosity of hyaluronate with nitrogen as gas-phase: methylene blue, K-indigo tetrasulphonate, K-indigo trisulphonate, K-indigo disulphonate, phenosafranine, rosinduline and neutral red.

Influence of hydrogen peroxide. Robertson *et al.* (1941) reported that addition of hydrogen peroxide to an ascorbic acid + hyaluronate mixture caused a 250-fold acceleration in the rate of loss of its characteristic precipitability, but McClean & Hale (1941),

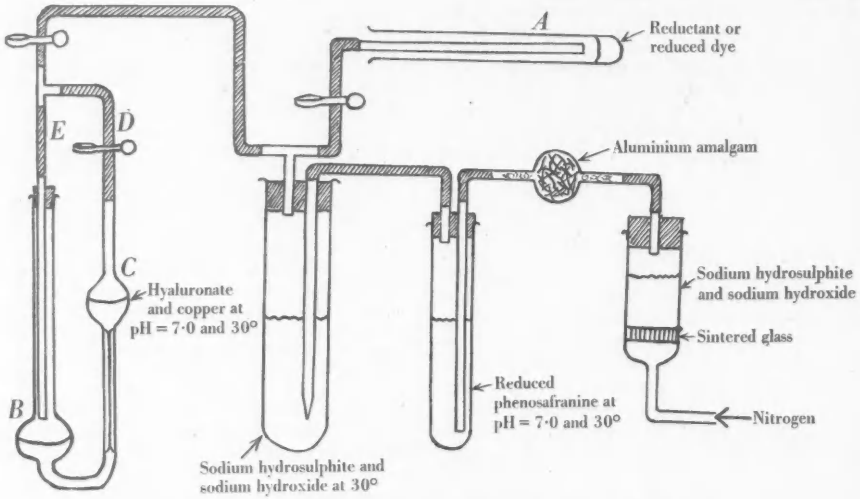


Fig. 2. Apparatus for viscosimetry in oxygen-free atmosphere.

When ascorbic acid (0·01 M, pH 7·0) was used in place of the dyes there was an initial slight fall in viscosity which was thereafter negligible until air was admitted, when the fall became rapid (Fig. 3).

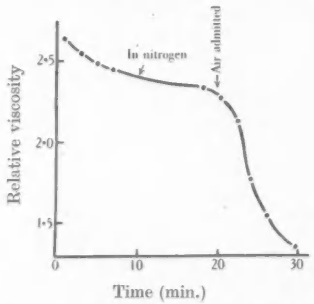


Fig. 3. Influence of oxygen on the viscosity of hyaluronate in the presence of ascorbic acid. 2·5 ml. buffered hyaluronate (0·25%) (pH 7·0). 0·5 ml. ascorbic acid (0·01 M).

With reduced phenosafranine the same result was observed on admitting oxygen. This need for oxygen partially explains the gradual nature of the degradation of the hyaluronate, since the rate will be affected by the accessibility of oxygen.

using a viscosimetric technique, found very little difference in the rate whether hydrogen peroxide was present or not. According to Barron, DeMeio

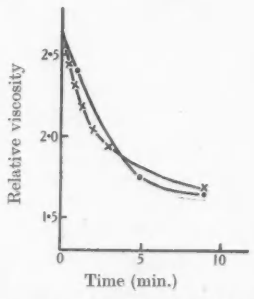


Fig. 4. Influence of catalase on the fall in viscosity of hyaluronate in the presence of ascorbic acid. •—• Water (1 vol.) + ascorbic acid + Cu + hyaluronate (11 vol.). ×—× Catalase (1 vol.) + ascorbic acid + Cu + hyaluronate (11 vol.). The catalase was still active at the end of the experiment.

& Klemperer (1935), hydrogen peroxide is formed during the catalytic oxidation of ascorbic acid by copper. Robertson *et al.* (1941) accordingly suggested that the degradation of hyaluronate by

ascorbic acid + copper might be due to hydrogen peroxide formation and reported that addition of catalase completely inhibited the degradation. We find that the addition of rabbit liver catalase to an ascorbic acid + Cu + hyaluronate mixture does not influence the rate of decrease of viscosity (Fig. 4). Furthermore, in an ascorbic acid + hyaluronate mixture to which excess hydrogen peroxide had been added, the addition of sodium diethyldithiocarbamate causes a marked decrease in the rate of lowering of viscosity whereas cupric sulphate accelerates it (Fig. 5). Since catalase did not inhibit this lowering

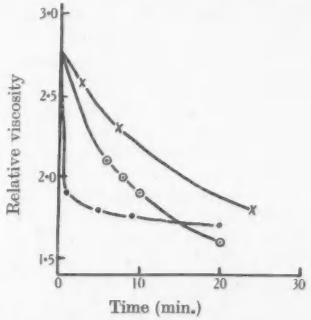


Fig. 5. Influence of Cu and Na diethyldithiocarbamate on the fall of viscosity of hyaluronate in the presence of ascorbic acid and excess H_2O_2. 2·5 ml. buffered hyaluronate and 0·5 ml. of a mixture of 1 vol. 0·01 M-ascorbic acid with 1 vol. 6% H_2O_2. •—• 0·1 mg. Cu (as sulphate) added. ×—× 1·0 mg. Na diethyldithiocarbamate added. ⊙—⊙ Nothing added.

of viscosity, hydrogen peroxide is apparently not essential. The inhibitory action of sodium diethyldithiocarbamate in the presence of excess hydrogen peroxide then indicates that the catalytic action of copper is due to some function other than the provision of hydrogen peroxide.

Influence of copper. Confirming Pirie (1942), we have found that the addition of cupric sulphate to an ascorbic acid + hyaluronate mixture increases the rate of degradation whereas the addition of sodium diethyldithiocarbamate inhibits the reaction. Sodium diethyldithiocarbamate similarly inhibited the catalytic action of copper upon the lowering of viscosity by cysteine or sodium sulphite. Even as much as 1 mg. of sodium diethyldithiocarbamate added to 3 ml. of the mixtures before the reducing agent does not result in a complete suppression of the change in viscosity but only in a very marked decrease in the rate.

Action of cuprous ion. In a mixture containing hyaluronate, cupric ions and reducing agent, cuprous ions might be the active agents in lowering viscosity. In the presence of air, saturated cuprous chloride (about 0·001 M) caused a rapid fall in the viscosity of hyaluronate; in nitrogen there was a very slight fall, but when air was subsequently admitted the viscosity fell rapidly (Fig. 6). Controls containing cupric chloride showed no fall in the presence or absence of oxygen. It seems therefore that cuprous ions together with oxygen will degrade

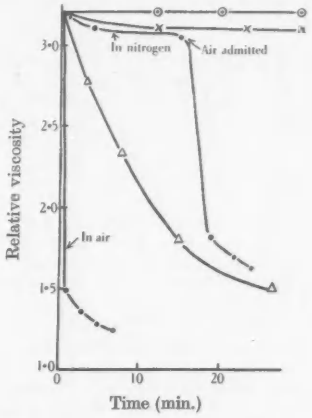


Fig. 6. Influence of oxygen on the viscosity of hyaluronate in the presence of cuprous chloride. Exp. 1. •—• Cu_2Cl_2 saturated (about 0·001 M) (0·5 ml.); 0·1 M buffer (0·5 ml.); 0·25% K hyaluronate (2 ml.). ⊙—⊙ $CuCl_2$ (0·001 M) (0·5 ml.); buffered hyaluronate solution (2·5 ml.). Exp. 2. ⊙—⊙ CO bubbled through saturated Cu_2Cl_2 solution for 1 hr. (0·5 ml.); buffered hyaluronate solution (2·5 ml.). ×—× 0·25% hyaluronate (2 ml.), and 0·1 M buffer (0·5 ml.), CO bubbled through 1 hr.; before addition of 0·01 M-ascorbic acid (0·5 ml.). △—△ 0·25% hyaluronate (2 ml.); 0·1 M-buffer (0·5 ml.); 0·00 M-ascorbic acid (0·5 ml.).

hyaluronate. Moreover, carbon monoxide which specifically combines with cuprous ions inhibits the effect. Cuprous chloride which had been exposed to carbon monoxide caused no fall in the viscosity of hyaluronate even when abundant oxygen was present.

Action of hydrogen in presence of catalytically active palladium. Hydrogen and palladium had no effect on the viscosity of hyaluronate in the absence of oxygen, but in its presence the viscosity of the hyaluronate and of starch was decreased.

The influence of phosphate. Since ascorbic acid + hydrogen peroxide will dephosphorylate β-glycerophosphate, their action on hyaluronate and other polysaccharides might be due to their action on a phosphate linkage (Robertson *et al.* 1941). We found that phosphorus-free hyaluronate (P = 0·002% on dry wt. of hyaluronate) was still viscous in aqueous solution and that its viscosity was decreased by exposure to ascorbic acid + oxygen (Fig. 7). The

addition of inorganic phosphate to the reacting mixtures did not alter the rate of decrease of viscosity. It seems unlikely, therefore, that ascorbic acid + copper + oxygen lower the viscosity by dephosphorylating hyaluronate.

Fig. 7. Influence of phosphate on the viscosity of P-free hyaluronate in the presence of ascorbic acid. P-free hyaluronate solution (0·5%) (5 ml.); ascorbic acid solution (0·01 M) (1 ml.); M-citric acid-phosphate buffer (0·6 ml.) (pH 7·0) added at 25 min.

Hyaluronate and the oxidation rate of ascorbic acid. The presence of hyaluronate had no influence on the rate of oxidation of ascorbic acid by oxygen.

DISCUSSION

It appears that the loss of viscosity which occurs when certain polysaccharides are exposed to the action of reducing agents takes place only when oxygen is present. Ascorbic acid, reduced phenosafranine, cuprous chloride or hydrogen must all be in a process of active oxidation in order to lower the viscosity of the polysaccharides. However, within the limits of the experimental method, potassium hyaluronate could not be shown to increase the rate of oxidation of ascorbic acid, although the polysaccharide was itself being altered. If the reducing agents used act primarily as reductants, then copper reduced to the cuprous state may be the active agent; as shown, cuprous copper with oxygen alone will produce a fall in viscosity. However, the catalytic action of copper in oxidation processes is well known, and in view of the results with ascorbic acid it seems more likely that it accelerates the polysaccharide degradation by increasing the rate of oxidation of the reducing agent. Ascorbic acid exposed to air becomes oxidized at pH 7·0 in the absence of copper, and it has been shown here that sodium diethyldithiocarbamate does not completely suppress the degradation of polysaccharide by reducing agents. The reaction will go on very slowly in the absence of copper which, however, catalyzes it.

Apparently the sole requirement in the mixture is a reducing agent undergoing active oxidation. It is of interest that such a process is capable of initiating several diverse reactions, e.g. the ascorbic acid + hydrogen peroxide dephosphorylation of glycerophosphate (Thannhauser, Reichel & Grattan, 1938; Robertson *et al.* 1941). Willison (1943) has shown that diphtheria toxin both undergoes inactivation and loses its antigenicity when exposed to sodium ascorbate when the ascorbate is undergoing reversible oxidation. The coupled oxidation of haemoglobin described by Lemberg, Legge & Lockwood (1939) may be a phenomenon of the same type. Lutwak-Mann (personal communication) has found that glucosamine and galactosamine, but not *N*-acetylglucosamine, undergo deamination in the presence of ascorbic acid + copper.

Bawn, Hirst & Young (1940) and Barker, Hirst & Young (1941) have obtained evidence, by end-group assay methods, that, in starch, the link between macromolecules, consisting of 25–30 glucose residues combined in 1:4 linkage, is a 1:6 glycoside linkage. If it be this link which is broken by the action of reducing agent and oxygen it is not easy to account for its instability compared with the stability of the 1:4 linkage. No evidence has yet been produced to show the nature of the linkages in hyaluronate, but it is of interest that similar reductant-oxygen systems will lower the viscosity of hyaluronate and starch. A study by end-group assay methods of the degradation products obtained by the action of reductant + oxygen systems upon these viscous polysaccharides might yield interesting results.

While this paper was being prepared for the press a communication by Skanse & Sundblad (1943) was received. These authors describe experiments very similar to some of those reported here and obtain substantially the same results. They conclude that the depolymerization of polysaccharides in the presence of molecular oxygen, copper and reducing agents is due to oxidation of the polysaccharide molecule. They support this by showing that during the depolymerization of hyaluronic acid by hydrogen peroxide or ascorbic acid there is an increase of titratable acid groups in an amount approximately corresponding to one carboxylic group for each disaccharide unit. They show that there is a destruction of the birefringence of flow exhibited by normal hyaluronic acid and that dialyzable substances containing nitrogen are produced. They suggest that a product of the reaction may be a disaccharide of glucuronic acid and *N*-acetylglucosaminic acid although they have not yet isolated such a compound. While they report that dehydroascorbic acid will degrade hyaluronate, they were

unable to show that this reagent was reduced to ascorbic acid during the action. Also they show that with dehydroascorbic acid the presence of molecular oxygen is also required to accomplish the degradation of the polysaccharides. These results, together with the observed inactivity of sodium sulphate, cystine, cupric ions and oxidized thiolacetic acid reported by us, throw some doubt on their suggestion that the oxidation products of the reducing agents directly oxidize the polysaccharide.

Experimental methods

K-hyaluronate from umbilical cord (McClean, 1943) was used throughout. The material (4·1% N) gave no biuret reaction but contained a small amount of a water-insoluble material containing iron, probably a haematin derivative (Humphrey, 1943). The polysaccharide was buffered at pH 7·0 with McIlvaine's (1921) citric acid + phosphate buffer to final conc. M/60 except where otherwise stated. Other methods employed included those for the estimation of *N*-acetylhexosamine (Morgan & Elson, 1934), *l*-ascorbic acid (Birch, Harris & Ray, 1933), Cu (Keilin & Mann, 1938), P (Martland & Robison, 1926). By the term relative viscosity is meant the flow-time of the solution divided by the flow-time of water in the same Ostwald viscosimeter at 30°. The solution in the viscosimeter was exposed to air except when the influence of some other gas was under examination (see below).

The influence of oxygen and various O/R *dyes on lowering of viscosity.* Nitrogen was passed through a series of reagents to remove oxygen in the apparatus shown in Fig. 2. A solution (0·01 M) of methylene blue or other dye was exactly reduced with $Na_2S_2O_4$ and 5 ml. of the reduced dye was pipetted into tube *A* shown in Fig. 2. Here it remained under an atmosphere of flowing nitrogen until it reached the temperature of the thermostat (30°). Meanwhile 2·5 ml. of a buffered hyaluronate solution (0·25%) were boiled and pipetted into the viscosimeter (*B*, Fig. 2) and allowed to remain under nitrogen until the temperature reached 30°. The reduced dye solution (0·5 ml.) was now transferred by pipette from tube *A* to the viscosimeter *B* and mixed with the hyaluronate by agitating the viscosimeter. The nitrogen flow to the viscosimeter was now stopped and the mixture forced from *B* into the viscosimeter bulb *C* by manipulating the rubber tubes *D*, *E* attached to the limbs of the viscosimeter. No reoxidation of the dye or fall in viscosity was observed during 18 hr.

The influence of catalase. Catalase was prepared from rabbit liver (Keilin & Hartree, 1936); 0·5 ml. of the preparation liberated approx. 7 ml. O_2/min. from 26 ml. of 0·46% H_2O_2 at pH 7·0 and 16°. This material was used in the experiment illustrated in Fig. 4.

The influence of hydrogen + palladium. Hyaluronate (4 ml. of 0·5% solution) was mixed with 1 ml. of M-buffer at pH 7·0 and 1 ml. of 1% $PdCl_2$. Hydrogen was bubbled through the mixture for 30 min. The relative viscosity before hydrogen was passed was 6·6: after hydrogen was passed it was 3·5. The rate of viscosity fall became slower until more hydrogen was passed, when the rate accelerated until the relative viscosity had fallen below 2. The pH at the end of the reaction was unchanged. In control experiments in which air was passed instead of hydrogen or hydrogen was used in the absence of $PdCl_2$, no fall in viscosity was observed. Hydrogen was passed through a solution of $PdCl_2$, the precipitate washed with water and suspended in hyaluronate solution. No fall in viscosity occurred until hydrogen was passed, when the viscosity fell rapidly. Copper, instead of palladium, will not catalyze this reaction.

The experiment was repeated in a hydrogenation apparatus, but the amount of hydrogen taken up per unit weight of polysaccharide was variable. Therefore an experiment was performed with an oxygen-free gas phase in the viscosimeter. Nitrogen was bubbled through a solution of hyaluronate containing activated palladium. When hydrogen was passed, very little fall in viscosity occurred until air was admitted, when the fall was rapid.

Experiments in which solutions of starch replaced the hyaluronate used in the foregoing experiments showed that this polysaccharide also is degraded by palladium + hydrogen + oxygen.

The influence of phosphate. A sample of purified K-hyaluronate (containing 1·25% P) after prolonged dialysis against distilled water contained only 0·232% P. Bone phosphatase (5 mg.) having primary phosphatase activity and a trace only of phosphodiesterase activity was added to 105 ml. of the dialyzed hyaluronate solution (pH 7·8) containing 500 mg. K-hyaluronate and 0·003 M-$MgCl_2$. The mixture was incubated at 37° for 6 hr. together with a control without phosphatase. After this time there was no difference in the viscosity of the two samples; both had a relative viscosity of 56. The phosphatase-treated sample was now dialyzed against frequent changes of 0·2 M-NaCl for 3 days, after which the P content was only 0·002% of the dry wt. of the hyaluronate. An indeterminable amount of this was inorganic. This phosphorus-free hyaluronate was used for the experiment illustrated in Fig. 7.

The influence of K-hyaluronate on the oxidation rate of ascorbic acid. A solution of hyaluronate (0·25%) saturated with chloroform, and a similar sample of chloroform water, were kept under identical conditions at 4°. To 4 ml. of each were added 1 ml. of buffer and 1 ml. of ascorbic acid (0·01 M). Both solutions were incubated at 34° and a sample of the hyaluronate + ascorbic acid mixture was observed for decrease of viscosity, which fell from 3·04 to 1·60 in 30 min. The ascorbic acid content of both samples after this period was 0·225 mg. ascorbic acid/ml.

SUMMARY

1. The influence of reducing agents on the viscosity and characteristic precipitability of various polysaccharides is described.

2. Molecular oxygen is essential to the action between these polysaccharides and the reducing agents used.

3. The influence of the reducing agents + oxygen on the polysaccharides is accelerated by the presence of cupric ions. Evidence is provided that the action of copper may be due to its reduction to the cuprous state.

4. Hydrogen in the presence of catalytically active palladium + oxygen will lower the viscosity of potassium hyaluronate and starch.

5. The bearing of these results on the general action of reducing agents on polysaccharides is discussed.

I wish to thank Dr D. McClean, in whose department this work was done, and Dr H. J. Rogers, for their constant interest and advice.

REFERENCES

Barker, C. C., Hirst, E. L. & Young, G. T. (1941). *Nature, Lond.*, **147**, 296.
Barron, E. S. G., DeMeio, R. H. & Klemperer, F. (1935). *J. biol. Chem.* **112**, 625.
Bawn, C. E. H., Hirst, E. L. & Young, G. T. (1940). *Trans. Faraday Soc.* **36**, 880.
Birch, T. W., Harris, L. J. & Ray, S. N. (1933). *Biochem. J.* **27**, 590.
Humphrey, J. H. (1943). *Biochem. J.* **37**, 460.
Keilin, D. & Hartree, E. F. (1936). *Proc. roy. Soc.* B, **121**, 173.
—— & Mann, T. (1938). *Proc. roy. Soc.* B, **125**, 187.
Lemberg, R., Legge, J. W. & Lockwood, W. H. (1939). *Biochem. J.* **33**, 754.
McClean, D. (1943). *Biochem. J.* **37**, 169.
— & Hale, C. W. (1940). *Nature, Lond.*, **145**, 866.
McClean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
Madinaveitia, J. & Quibell, T. H. H. (1941). *Biochem. J.* **35**, 456.
Martland, M. & Robison, R. (1926). *Biochem. J.* **20**, 847.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Pirie, A. (1942). *Brit. J. exp. Path.* **23**, 277.
Robertson, W. van B., Ropes, M. W. & Bauer, W. (1940). *J. biol. Chem.* **133**, 261.
—— —— —— (1941). *Biochem. J.* **35**, 903.
Seshagirirao, P. & Giri, K. V. (1942). *Proc. Indian Acad. Sci. Bangalore*, **16** B, 190.
Skanse, B. & Sundblad, L. (1943). *Acta phys. Scand.* **6**, 37.
Thannhauser, S. J., Reichel, M. & Grattan, J. F. (1938). *Biochem. J.* **32**, 1163.
Willison, F. E. (1943). *J. Immunol.* **47**, 409.

Studies on Diffusing Factors

5. THE INFLUENCE OF SOME ENVIRONMENTAL CONDITIONS ON THE ACTIVITY OF HYALURONIDASE

By C. W. HALE, *The Lister Institute of Preventive Medicine, Elstree, Herts*

(*Received* 19 *February* 1944)

In the course of other work (Hale, 1944), observations were made to determine whether reducing agents or other substances which affect the viscosity of hyaluronic acid also exert any influence on the activity of hyaluronidase.

METHODS

The methods used were those described previously (McClean & Hale, 1941; McClean, 1943; Hale, 1944).

RESULTS

Influence of oxygen on hyaluronidase activity. Experiments were carried out in the viscosimeter apparatus described by Hale (1944). There was no significant difference in the activity of the enzyme under any of the conditions shown in Table 1. It is concluded that the enzyme does not need molecular oxygen for its activity, nor does the enzyme during its action on the substrate at pH 7·0 produce conditions which will either oxidize reduced rosinduline or reduce indophenol. The activity of the enzyme is not readily affected by exposure to oxygen.

Influence of Cu *and* Na *diethyldithiocarbamate on hyaluronidase.* Since Cu increases the rate of reaction in a reductant + oxygen + hyaluronate mixture it was thought that it might have a similar action upon hyaluronidase + hyaluronate. Copper (0·1 mg. as sulphate) was added to 2 ml. hyaluronate (0·25 %) + 0·5 ml. of 0·1 M-McIlvaine's buffer (pH 7·0) + 0·5 ml. *Cl. welchii* hyaluronidase dilution,

Table 1. *The influence of oxygen on hyaluronidase activity*

(The enzyme + substrate mixture had the following composition:
2·0 ml. K-hyaluronate (0·25 %) } previously boiled
0·5 ml. McIlvaine's buffer (pH 7·0) } and cooled.
0·5 ml. crude *Cl. welchii* hyaluronidase (1:100 in boiled and cooled water).
Temperature of thermostat 30°.
Rosinduline, exactly reduced with $Na_2S_2O_4$, included as a reducing agent and as an indicator of oxidation.
Indophenol added as an indicator of reduction.)

Conditions	v.r.u.* activity	Remarks
In nitrogen + trace of reduced rosinduline	125	Rosinduline not re-oxidized
,,	105	,,
In nitrogen + trace of indophenol	100	Indophenol not reduced
In air; no dye	108	—
,,	96	—
In oxygen; enzyme and substrate saturated with oxygen	95	—

* v.r.u. = viscosity reducing units (McClean, 1943).

10 min. after the viscosity fall had begun. No change in the rate of fall of viscosity was observed after adding the copper. In another experiment Na diethyldithiocarbamate (1 mg.) was added to the usual enzyme + substrate mixture. Again no change in rate of fall of viscosity was observed. It is concluded that copper plays no part in the activity of hyaluronidase.

Influence of oxidizing agents on hyaluronidase activity. Meyer, Dubos & Smyth (1937) and, in confirmation, Meyer, Chaffee, Hobby & Dawson (1941), reported that hyaluronidase prepared from an autolysate of a type I *Pneumococcus* was inactivated by iodine and could be reactivated by arsenite or sulphite. Robertson, Ropes & Bauer (1940) found that hyaluronidase from a culture of *Cl. welchii* was also inactivated by iodine, but were unable to reverse the inactivation. In view of these contradictory reports, and of the relative insusceptibility of *Cl. welchii* hyaluronidase to oxygen, the matter was investigated by means of the viscosimetric assay method. It was found that crude hyaluronidase from *Cl. welchii* and hyaluronidase from testis purified by the method of Madinaveitia (1941) were both partially inactivated by $KMnO_4$ and by iodine (0·001–0·0001 M). It was found that the testis enzyme (dry wt. 5·55 mg./ml.; activity 180 V.R. units/mg.) (McClean, 1943) was not completely inactivated when 0·001 M-iodine was used. This was an excess of iodine for, after 30 min. exposure, free iodine could still be detected. Each enzyme preparation, partially inactivated by either iodine or $KMnO_4$, was not reactivated by $Na_2S_2O_4$.

Influence of pH *on streptococcal hyaluronidase.* The anomalous behaviour of hyaluronidase derived from haemolytic streptococci when examined viscosimetrically has been reported by Meyer *et al.* (1941), and McClean (1941). This behaviour of these enzyme preparations was of importance, since it prevented their assay by the viscosimetric method then in use. Madinaveitia & Quibell (1940) stated that, under defined conditions of viscosimetric assay, samples of hyaluronidase derived from mammalian testis decrease the viscosity of hyaluronic acid at a rate directly proportional to the enzyme concentration. The enzymes from *Cl. welchii*, *Cl. septicum* and *Staph. aureus* behave similarly (McClean & Hale, 1941). The composition of the mixture which was used by McClean & Hale (1941) was as follows: 4 ml. of a 0·25 % hyaluronate solution, 1 ml. M-Na citrate (pH 4·6) containing 0·6 M-NaCl and 1 ml. of enzyme dilution in distilled water. Under these conditions, hyaluronidase from group A and C streptococci did not behave in the same quantitative way as the enzymes from other sources. An example of the effect observed is illustrated in Fig. 1. Whereas a 1:10 dilution of the enzyme reduced the viscosity of the system to half in 10 min., a 1:20 dilution did not cause this reduction in 50 min., and a 1:40 dilution barely caused any decrease in viscosity. The enzyme appeared to be destroyed or inhibited during its action. It has been found (McClean, 1943) that testicular and bacterial enzymes show

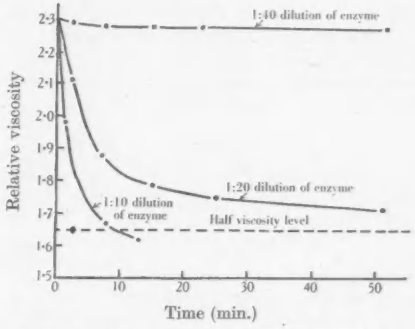


Fig. 1. The activity of three concentrations of group A streptococcal hyaluronidase in 0·16 M-Na citrate at pH 4·6.

their optimum activity in a system containing 1 vol. of McIlvaine's 0·1 M-citric acid-phosphate buffer (pH 7·0) instead of the 1 vol. of M-Na citrate (pH 4·6) previously used. For this reason the technique for viscosimetric assay was modified so that the reaction

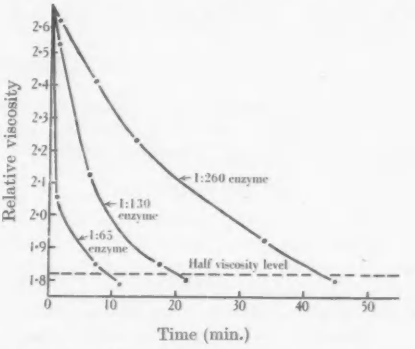


Fig. 2. The activity of three concentrations of group A streptococcal hyaluronidase in 0·016 M-McIlvaine buffer at pH 7·0.

of the system was now pH 7·0, instead of pH 4·6, and the salt content decreased. When streptococcal hyaluronidase was tested in this way it caused a normal viscosity fall (Fig. 2). The enzyme sample used in this experiment was the same as that used to obtain the values of Fig. 1. Several experiments were performed using Na-citrate buffer (pH 4·6) in concentrations varying from 0·16 to 0·016 M. Exposure of streptococcal hyaluronidase to this buffer

before it is mixed with the substrate results in complete inactivation of the enzyme. Similar results are obtained if citric acid-phosphate buffer is used at pH 4·6; if this pH is employed in the viscosimetric assay of streptococcal hyaluronidase, abnormal curves are always obtained and no quantitative assays can be made. It appears that hyaluronidase from either group A or group C streptococci differs from the enzymes obtained from testis, *Cl. welchii* and staphylococci in that it is rapidly destroyed at pH 4·6, but is relatively stable at pH 7·0.

SUMMARY

1. Hyaluronidase from *Cl. welchii* is not readily inactivated by molecular oxygen, does not require oxygen for its activity and does not produce conditions which will either oxidize reduced rosinduline or reduce indophenol during its activity on the hyaluronate at pH 7·0.

2. The presence or absence of Cu has no influence on the activity of hyaluronidase from *Cl. welchii*.

3. Hyaluronidase derived either from *Cl. welchii* or testis is partially inactivated by $KMnO_4$ and by iodine; this inactivation is not reversed by $Na_2S_2O_4$.

4. Hyaluronidase derived either from group A or group C streptococci is rapidly inactivated at pH 4·6, but is relatively stable at pH 7·0. This inactivation explains previous reports of the anomalous viscosimetric behaviour of streptococcal hyaluronidase.

REFERENCES

Hale, C. W. (1944). *Biochem. J.* **38**, 362.
McClean, D. (1941). *J. Path. Bact.* **53**, 13.
—— (1943). *Biochem. J.* **37**, 169.
—— & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
Madinaveitia, J. (1941). *Biochem. J.* **35**, 447.
—— & Quibell, T. H. H. (1940). *Biochem. J.* **34**, 625.
Meyer, K., Dubos, R. & Smyth, E. M. (1937). *J. biol. Chem.* **118**, 71.
—— Chaffee, E., Hobby, G. L. & Dawson, M. H. (1941). *J. exp. Med.* **73**, 309.
Robertson, W. van B., Ropes, M. W. & Bauer, W. (1940). *J. biol. Chem.* **133**, 261.

The Antibacterial Action of some Diphenyl Derivatives

By G. H. FAULKNER, *The Northumberland County Laboratory, Newburn-on-Tyne*

(*Received* 15 *August* 1944)

The observations recorded in this paper are a continuation of those previously reported; similar methods have been used (Faulkner, 1943, 1944).

EXPERIMENTAL

The anti-bacterial test. In the standard experiment organisms are incubated at 37° in broth containing the substance being investigated, and subcultured on to solid media after 24 and 48 hr. to test survival. To test bacteriostatic action the organisms are sown on to agar-medium in which the bactericide is incorporated in a concentration 1:2500.

Organisms used. All the organisms used were freshly isolated from material submitted for bacteriological examination, and were as follows: *Staph. aureus*, three strains; haemolytic *Streptococcus* Group A, three strains; *C. diphtheriae*, four strains all virulent to guinea-pigs; *Diplococcus pneumoniae*, two strains; *Streptococcus faecalis*, three strains; Gram-negative diplococci, two strains; *Klebsiella pneumoniae* (Friedlaender's bacillus); and seven strains of Gram-negative intestinal bacilli: *Esch. coli*, *Shigella paradysenteriae*, *Shigella sonnei* (*B. dysenteriae* Sonne), *Salmonella typhimurium* (*B. aertrycke*), *Salmonella* sp. *oranienburg* type, *Salmonella schottmuelleri* (*Bact. paratyphosum* B) and *Eberthella typhosa*.

Compounds used. The starting-point of this series of experiments was diethylstilboestrol, i.e. 4-OH.C_6H_4CEt:CEt.C_6H_4.OH-4′. Most of the substances tested contain two benzene rings; a few compounds were included containing three benzene rings, or only one, but none of these showed any antibacterial activity. Many of the compounds are not soluble in the concentrations used. In these cases they were used as a fine suspension obtained by adding an ethanol solution to broth.

RESULTS

Table 1 summarizes the results of experiments with the most active compounds. Such a compilation does not give a complete picture, as it does not take into account the number of different organisms against which the substance is active, or the variations in the relative susceptibilities of the organisms to the compounds. In general the order of decreasing susceptibility is: *C. diphtheriae*; haemolytic *Streptococcus* and *Diplococcus pneumoniae* approxi-

Table 1. *List of most active compounds related to diphenyl arranged approximately in order of diminishing bactericidal activity*

Compound	Groups separating the rings	Groups attached to the rings	Greatest dilution at which bactericidal
1. 4-Hydroxy-α:β-diethylstilbene	—CEt:CEt—	OH	1 : 600,000
2. 4:4′-Dihydroxy-α:β-diethylstilbene (diethylstilboestrol)	—CEt:CEt—	OH OH	1 : 500,000
3. 4:4′-Dihydroxy-γ:δ-diphenylhexane (hexoestrol)	—CHEt.CHEt—	OH OH	1 : 500,000
4. 4:4′-Dihydroxy-α-*iso*propylstilbene	—CC_3H_7:CH—	OH OH	1 : 100,000
5. 4:4′-Dihydroxy-α-*n*-propylstilbene	—CC_3H_7:CH—	OH OH	1 : 100,000
6. 3:3′-Dihydroxydiphenylhexadiene	—$CC_2H_4.CC_2H_4$—	OH OH	1 : 50,000
7. Diazoaminobenzene	—$N_2.NH$—	—	1 : 50,000
8. 4:4′-Dihydroxydiphenylhexadiene (dienoestrol)	—$CC_2H_4.CC_2H_4$—	OH OH	1 : 50,000
9. 4-Hydroxystilbene	—CH:CH—	OH	1 : 50,000
10. *o*-Benzylphenol	—CH_2—	OH	1 : 10,000
11. *p*-Benzylphenol	—CH_2—	OH	1 : 10,000
12. *o*-Hydroxydiphenyl	—	OH	1 : 10,000
13. *p*-Hydroxydiphenyl	—	OH	1 : 10,000
14. Diphenylamine	—NH—	—	1 : 10,000
15. Diphenylmethane	—CH_2—	—	1 : 10,000
16. Diphenylether	—O—	—	1 : 10,000
17. 4:4′-Dihydroxydiphenyl	—	OH OH	1 : 10,000
18. 3:3′-4:4′-Tetrahydroxy-γ:δ-diphenyl-*n*-hexane	—CHEt.CHEt—	OH OH OH OH	1 : 10,000
19. Dibenzylether	—$CH_2.O.CH_2$—	—	1 : 10,000
20. 2:4-Diaminoazobenzene (chrysoidine)	—N:N—	NH_2 NH_2	1 : 10,000
21. Diphenyl	—	—	1 : 10,000
22. 4:4′-Dihydroxy-α-ethylstilbene	—CEt:CH—	OH OH	1 : 10,000
23. Dimethoxy-α:β-diethylstilbene	—CEt:CEt—	CH_3O CH_3O	1 : 10,000
24. 4:4′-Dihydroxy-β:γ-diphenylbutane	—$CHCH_3.CHCH_3$—	OH OH	1 : 10,000

Table 2. *Comparative activities of the five most active diphenyl compounds for various susceptible organisms*

Organism	Compound: 4-Hydroxy-α:β-diethylstilbene	Diethylstilboestrol	Hexoestrol	4:4′-Dihydroxy-α-*iso*propylstilbene	4:4′-Dihydroxy-α-*n*-propylstilbene
	Greatest dilution at which bactericidal				
C. diphtheriae	1 : 600,000	1 : 200,000	1 : 500,000	1 : 100,000	1 : 100,000
Streptococcus pyogenes	1 : 400,000	1 : 300,000	1 : 100,000	—	—
Diplococcus pneumoniae	1 : 100,000	1 : 50,000	1 : 100,000	—	—
Staphylococcus aureus	1 : 250,000	1 : 50,000	1 : 50,000	1 : 10,000	1 : 10,000
Streptococcus faecalis	—	1 : 50,000	1 : 50,000	1 : 10,000	1 : 10,000
Neisseria catarrhalis	—	1 : 500,000	—	—	—
Neisseria pharyngis	1 : 5,000	1 : 5,000	—	—	—

mately similar; *Staph. aureus*; *Streptococcus faecalis*. Many of the substances were bacteriostatic to organisms which they did not kill. The effect on Gram-negative organisms is discussed later.

Of all the substances tested the only one superior to diethylstilboestrol and hexoestrol is 4-hydroxy-α:β-diethylstilbene. These three compounds have a marked superiority over all others tested and are bactericidal to at least one type of organism in a dilution of 1 : 500,000 (see Table 2).

In addition to the compounds listed in Table 1 the following were bactericidal in concentrations between 1 : 10,000 and 1 : 2500:

(25) stilboestrol dipropionate, (26) dibenzylamine, (27) 4:4′-dihydroxy-stilbene, (28) hexoestrol dimethyl ether, (29) benzidine, (30) hydrazobenzene, (31) azobenzene, (32) di-*p*-tolylamine, (33) dibenzyl, (34) dibenzyl ketone.

The following compounds showed neither bactericidal nor bacteriostatic action in a concentration of 1 : 2500:

m-diphenylbenzene; *p*-diphenylbenzene; phenylmethyl ether; phenylethyl ether; benzylmethyl ether; benzylethyl ether; diphenylamine sulphonic acid; barium diphenylamine sulphonate; (sym) diphenyl urea; triphenylamine; triphenylmethane; phenyl-ethylene; triphenyl-ethylene; triphenyl-chloro-ethylene; diphenyl-ethylene (stilbene); stilbene dibromide; 2:2′-dihydroxy-α:β-diethyl-stilbene; 4:4′-dimethoxy-α:β-dichloro-stilbene; and stilboestrol dibutyrate.

DISCUSSION

The one feature common to all the active compounds tested is the presence of two benzene rings. These rings may be separated by one or more radicals and may have one or more radicals attached to

themselves. But diphenyl itself possesses weak bactericidal properties, being lethal to pneumococci in a dilution of 1:10,000 and to three strains of *C. diphtheriae* in a dilution of 1:2500.

The most active compounds contain one or more hydroxyl groups (i.e. the phenolic structure) but this is not essential. One OH group is better than two OH groups in the following pairs of compounds: nos. 1 and 2; 12 or 13 and 17; also 9 and 27. This is not evident from the data given in Table 1 but is apparent when detailed results are compared: for instance *p*-hydroxy-diphenyl is lethal to *Staph. aureus* in a concentration of 1:10,000, but dihydroxy-diphenyl is only bacteriostatic to this organism in a concentration of 1:2500. Comparison of nos. 18 and 3 shows that increasing the number of hydroxyls from two to four decreases the activity.

The position of the hydroxyl groups makes some difference: if the hydroxyls of diethylstilboestrol are transferred to position 2 the resulting compound is quite inactive. Two compounds containing a propyl group (4 and 5) are more effective than the corresponding compound containing an ethyl group (22). Also two ethyl groups (3) are better than two methyl groups (24).

Of all the compounds tested only five exert any inhibition on the growth of the Gram-negative intestinal bacilli (Table 3). It is noteworthy that *p*-hydroxy-diphenyl appears to differentiate between the Salmonellae and the other Gram-negative bacilli.

The bactericidal action of diethylstilboestrol and of 4-hydroxydiethylstilbene on tubercle bacilli is the subject of a separate communication (Faulkner, 1944).

Some of the compounds used possess oestrogenic activity, but there seems to be no correlation between the degree of bactericidal activity and their oestrogenic properties as recorded by Emmens (1941, 1942). The present observations confirm those of Brownlee, Copp, Duffin & Tonkin (1943), who used some 30 stilbene derivatives, four of which were tested in the present experiments. The best compound found by these authors is the same as that recorded above, namely, 4-hydroxydiethylstilbene.

SUMMARY

1. Bactericidal activity is recorded in 34 compounds of the diphenyl or stilbene type.

2. Five compounds are active in a concentration of 1:100,000 or less; the most efficient is 4-hydroxy-α:β-diethylstilbene.

3. These substances are most active against Gram-positive organisms; the susceptibility decreases in the order: *C. diphtheriae, Diplococcus pneumoniae*, haemolytic streptococci, *Staphylococcus aureus, Streptococcus faecalis*.

4. Only five substances were found to exert any inhibitory action on the Gram-negative intestinal bacilli tested.

5. The most efficient bactericides contain one or more hydroxyl groups in the molecule, but this configuration is not essential.

Table 3. *Comparative activities of the five compounds active against the intestinal Gram-negative bacilli*

(The compounds were dissolved or suspended in nutrient broth. Bacteriostatic action was tested in a dilution of 1:2,500.)

Organism	Compound: *o*-Benzyl-phenol	*p*-Benzyl-phenol	*o*-Hydroxy-diphenyl	*p*-Hydroxy-diphenyl	Diphenyl-amine
	Greatest dilution at which bactericidal				
Esch. coli	1:10,000	1:5,000	1:5,000	1:2,500	Bacteriostatic
Shigella paradysenteriae	1: 5,000	1:5,000	1:5,000	1:2,500	1:5,000
Shigella sonnei	1: 5,000	1:5,000	1:5,000	1:2,500	1:2,500
Salmonella typhimurium	1: 5,000	1:5,000	1:5,000	Bacteriostatic	Bacteriostatic
Salmonella sp. *oranienburg*	1: 2,500	1:2,500	1:5,000	None found	None found
Eberthella typhosa	1: 5,000	1:5,000	1:5,000	Not tested	1:2,500
Salmonella schottmuelleri	1: 5,000	1:2,500	1:5,000	None found	Bacteriostatic
Klebsiella pneumoniae	1: 5,000	1:2,500	1:5,000	1:2,500	None found

I am indebted to Prof. E. C. Dodds for compounds nos. 1, 6, 8, 9, 18, 22, 27, and 2:2′-dihydroxy-α:β-diethylstilbene; to Sir Robert Robinson for nos. 4, 5, 24, also 4:4′-dimethoxy-α:β-dichlorostilbene; and to Boots Pure Drug Co. Ltd. for eight compounds. I express my thanks to Dr A. I. Messer for continued help during this investigation, and to Prof. J. H. Gaddum for much advice both in planning and in interpreting the experiments.

REFERENCES

Brownlee, G., Copp, F. C., Duffin, W. M. & Tonkin, I. M. (1943). *Biochem. J.* **37**, 572.
Emmens, C. W. (1941). *J. Endocrinol.* **2**, 444.
Emmens, C. W. (1942). *J. Endocrinol.* **3**, 168.
Faulkner, G. H. (1943). *Lancet*, **2**, 38.
—— (1944). *Amer. Rev. Tuberc.* **50**, 167.

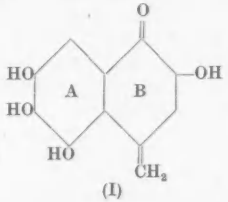
The Colouring Matters of Galls

By M. NIERENSTEIN and A. SWANTON, *Biochemical Laboratory, University of Bristol*

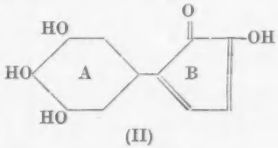
(*Received* 18 *September* 1944)

Dryophantin, $C_{23}H_{28}O_{15}$, the pigment of the 'Red Pea' gall, produced by *Dryophanta divisa* Adl. on the leaves of *Quercus pedunculata* Ehrh., yields on hydrolysis with dilute sulphuric acid 1 mol. of purpurogallin, $C_{11}H_8O_5$, and 2 mol. of glucose (Nierenstein, 1919). Extension of this work to other galls, produced either by insects or fungi, gave identical results, only whereas dryophantin is red, some of the pigments were differently coloured. Since some of them were green we examined each pigment spectroscopically for chlorophyll and also tested qualitatively for nitrogen. The results were negative, which thus excludes contamination with chlorophyll.

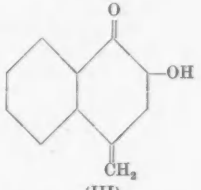
Although purpurogallin was first obtained a hundred years ago by Buchner (1847) it is still somewhat of a chemical enigma. Two structural formulae have been suggested for purpurogallin, namely, Formula (I) by Dean & Nierenstein (1913) and Formula (II) by Willstätter & Heiss (1923), neither of which, however, fully accounts for the chemical behaviour of purpurogallin, as already pointed out by Nierenstein (1934). Speculations as to the causes of these differences in colour of these glucosides of purpurogallin appear to us at the present premature and must be left open until purpurogallin has been synthesized. As work in this direction is in progress we would like to mention an observation made by Dr N. Green in 1928, which to some extent seems to undermine the validity of the Willstätter formula. Purpurogallin is known to yield on distillation with zinc dust naphthalene (Nietzki & Steinmann, 1887; Hooker, 1887; Perkin & Steven, 1903; Nierenstein & Spiers, 1913), and this is also the case when substance (III) is submitted to distillation with zinc dust (Dean & Nierenstein, 1916). According to Green, whose work will be published in due course, no trace of naphthalene is produced when substance (IV) is distilled with zinc dust:



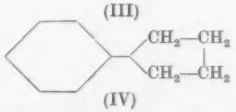
(I)



(II)



(III)



(IV)

Whatever ultimately the constitution of purpurogallin proves to be, there is no doubt whatever that it contains four hydroxyl groups, so that four isomeric biosides and six isomeric di-monosides are possible, thus giving a total of ten possible glucosides. The present communication describes eight such glucosides, named according to the gall producer, the method already adopted in the case of dryophantin.

EXPERIMENTAL

This investigation has been in progress from 1921 to 1942, with the result that after many experiments we have elaborated the following general method: (1) The materials, dried at 100°, were milled and percolated with ethanol followed by chloroform and carbon tetrachloride so as to remove chlorophyll, waxes, *cyclo*gallipharic acids, etc. We found that percolation with the individual solvents does not give easily workable materials. After these solvents had evaporated, the dry powder, mixed with ten times its weight of dry silver sand, was exhaustively extracted in a Soxhlet with boiling water until the overflow became colourless. On evaporation to about one-third of its volume, the solution crystallized on standing in the laboratory or on ice. The products thus obtained were several times crystallized from water until the melting-points became constant. All pigments gave the colour reactions previously described by Nierenstein (1919). (2) Since, as previously observed, hydrolysis with sulphuric acid gives lower values for purpurogallin than theoretically required (Nierenstein, 1919), even if corrections for purpurogallin are made according to Willstätter & Stoll (1918), we used enzymic

hydrolysis with the purpurogallase already described by Nierenstein & Thomas (1941). (3) The difficulties in obtaining reliable melting-points for purpurogallin have already been dealt with by Nierenstein & Spiers (1913), and we have therefore identified it as tetraacetylpurpurogallin, which has a sharp melting-point. (4) The glucose residue was identified as the osazone and quantitatively estimated according to Geake & Nierenstein (1914). (5) All molecular weight estimations were made in ethanol. (6) For the identification of galls and gall producers, we used Appel (1899), Connald (1909), Ross (1911, 1916) and Böhner (1935). (7) The yields are poor and the maximum yield obtained by us was 0·83%.

ANALYTICAL AND DESCRIPTIVE DATA

(1) *Adelgesin.* Light brown needles, m.p. 204–206°, isolated from the 'Pineapple' gall produced by *Adelges abietes* L. on the bark of *Abies excelsa* de Cond. (Found: C, 50·61; H, 5·27; purpurogallin, 40·23; glucose, 63·32%; molecular weight (m.w.), 538, 541. $C_{23}H_{28}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·37; glucose, 63·21%; m.w., 544.) From the 'April bud' gall produced on the buds of *Quercus* species by *Spathegaster aprilinus* Giz., yellowish green needles were obtained which had the same melting-point and composition as adelgesin; a mixed melting-point showed no depression.

(2) *Dryophantin.* Terra-cotta coloured needles, m.p. 220–221°, from the 'Red Pea' gall, produced by *Dryophanta divisa* Adl. on the leaves of *Quercus pedunculata* Ehrh. and from the 'Cherry' gall produced by *Dryophanta Taschenbergii* Schulz on the leaves of *Quercus sessiliflora* Sal. (Found: C, 50·61; H, 5·21; purpurogallin, 40·26; glucose, 63·21%; m.w. 544. $C_{23}H_{28}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·37; glucose, 63·21%; m.w. 544.) From the 'Two-cell' gall produced by *Dryophanta disticha* Htg. on the shoots of various species of *Quercus*, dull orange-coloured needles, and the gall produced by *Asphondolia genistae* Lew. on the leaves and stems of *Genista tinctoria* L., brown-coloured needles, which had the same melting-point and composition as dryophantin; mixed melting-points showed no depressions.

(3) *Cecidomin.* Flame-coloured orange needles, m.p. 227–231°, produced by *Cecidomyia tiliae* Schk. on the twigs and young shoots of *Tilia vulgaris* L. (Found: C, 50·69; H, 5·21; purpurogallin, 40·16; glucose, 63·18%; m.w. 556, 561. $C_{23}H_{28}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·37; glucose, 63·21%; m.w. 544.) From the 'Lighthouse' gall produced by *Cecidomyia bursaria* Bret. on the leaves of *Nepeta hedracea* Ter. and the 'Hedgebank' gall, produced by *Xestophanus potentillae* Cam. on the leaves of *Potentilla reptans* L., both of which crystallized in orange-coloured needles, which had the same melting-point and composition as cecidomin; mixed melting-points showed no depression.

(4) *Melampsorin.* Pale yellow needles, m.p. 233–237°, produced by *Melampsora goepportiana* Htg. on the leaves and roots of *Vaccinium vitis Idaea* L. (Found: C, 50·68; H, 5·22; purpurogallin, 40·41; glucose, 63·41%; m.w. 531, 549. $C_{23}H_{28}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·37; glucose, 63·21%; m.w. 544.) From the 'Pitmaking' gall produced by *Eriophyes ajugae* Nal. on the leaves of *Ajuga reptans* L., dark yellow needles which had the same melting-point and composition as melampsorin; a mixed melting-point showed no depression.

(5) *Raestelin.* Bright green needles, m.p. 242–244°, isolated from the gall produced by *Raestelia lucerta* Sbg. on the twigs of *Caraegus oxycanta* L. (Found: C, 50·67; H, 5·24; purpurogallin, 40·37; glucose, 63·27%; m.w. 526, 541, 549. $C_{23}H_{18}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·37; glucose, 63·21%; m.w. 544.)

(6) *Eriophyesin.* Brownish red needles, m.p. 257–261°, produced by *Eriophyes macrotrichus* Nal. on the leaves of *Caprinus betulus* L. (Found: C, 50·71; H, 5·17; purpurogallin, 40·22; glucose, 63·27%; m.w. 531, 543. $C_{23}H_{18}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·37; glucose, 63·21%; m.w. 544.) From the 'Nail' gall produced by *Eriophyes gracilis* Nal. on the leaves of *Rubus idaeus* L., dark red needles; the 'Witches Broom' produced by *Exoascus turgidus* Sad. on the twigs of *Betula alba* L., dark green needles; the 'Bean' gall produced by *Nematus gallicola* Staf. on the leaves of *Salix fragilis* L., light green needles; the 'Blister' gall produced by *Nematus Bridgemanii* Cam. on the leaves of *Salix caprae* L., dark green needles, and the 'Cherry' gall produced by *Neuroterus lenticularis* Oliv. on the leaves of *Quercus pedunculata* Ehrh., golden plates. All of these had the same composition as eriophyesin, the same melting and mixed melting-points.

Eriophyesin on methylation with diazomethane and hydrolysis yields 2:3:4-trimethoxypurpurogallin (Perkin & Steven, 1903; Willstätter & Heiss, 1923; Evans & Dehn, 1930) identified by its melting and mixed melting-points with authentic 2:3:4-trimethoxypurpurogallin. In both formulae I or II nucleus (A) is the same, which thus goes to prove that in eriophyesin the biglucose residue is attached to the hydroxyl group in nucleus (B).

(7) *Xestophanesin.* Bronze-coloured needles, m.p. 263–266°, isolated from the 'Hedgebank' gall produced by *Xestophanus potentillae* Cam. on the leaves of *Potentilla reptans* L. (Found: C, 50·49; H, 5·31; purpurogallin, 40·29; glucose, 63·18%; m.w. 537, 546, 557. $C_{23}H_{18}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·37; glucose, 63·21%; m.w. 544.)

(8) *Pontanin.* Orange-yellow needles, m.p. 281–287°, isolated from the galls produced by *Pontania proxima* Lepel on the leaves of *Salix fragilis* L. (Found: C, 50·61; H, 5·23; purpurogallin, 40·31; glucose, 63·11%; m.w. 542, 553. $C_{23}H_{18}O_{15}$ requires: C, 50·73; H, 5·15; purpurogallin, 40·31; glucose, 63·21%; m.w. 544.) From the 'Cotton-wool' gall produced by *Eriophyes Thomasii* Nal. on the leaves of *Thymus serpyllum*, blood red needles; from the 'Cupped Spangle' gall produced by *Neuroterus fumipennis* Hartg. on the leaves of *Quercus sessiliflora* Sal., golden plates; from the 'Smooth Pea' gall produced by *Rhodites eleganteriae* Hartg. on the leaves of *Rosa canina* L., yellow needles, and from the 'Fig' gall produced by *Pemphigus ulmi* de Geer on the bark of *Ulmus campestris* L., dark brown needles. All of these had the composition and melting-point of pontanin and showed no depression on admixture with it.

SUMMARY

1. The isolation and characterization of eight colouring matters from various galls is described.

2. All the pigments described were found to be diglucosides of purpurogallin. In the case of one of them (eriophyesin) it is shown that the biglucoside residue is attached to the hydroxyl group of the 'B' nucleus in purpurogallin.

We wish to thank Miss K. W. Sullvin, B.Sc., for the description of these pigments and for the trouble she has taken in comparing our dryophantin with the original dryophantin, which she has found to be identical in every respect.

REFERENCES

Appel, O. (1899). *Ueber Phyto- und Zoomorphosen (Pflanzengallen)*. Königsberg: R. Lempold.
Böhner, K. (1935). *Geschichte der Cecidologie*. Mittelwalde (Bayern): A. Nemayer.
Buchner, J. A. (1847). *Liebigs Ann.* **53**, 357.
Connald, E. F. (1909). *Plant Galls of Great Britain*. London: Adlard and Son.
Dean, H. F. & Nierenstein, M. (1913). *Ber. dtsch. chem. Ges.* **46**, 3868.
—— —— (1916). *J. chem. Soc.* **109**, 593.
Evans, T. W. & Dehn, W. M. (1930). *J. Amer. chem. Soc.* **52**, 3649.
Geake, A. & Nierenstein, M. (1914). *Ber. dtsch. chem. Ges.* **47**, 893.
Hooker, S. C. (1887). *Ber. dtsch. chem. Ges.* **20**, 3259.
Nierenstein, M. (1919). *J. chem. Soc.* **115**, 1328.
—— (1934). *The Natural Organic Tannins*. London: J. and A. Churchill.
—— & Spiers, C. W. (1913). *Ber. dtsch. chem. Ges.* **46**, 3151.
—— & Thomas, J. C. (1941). *Analyst*, **66**, 789.
Nietzki, M. & Steinmann, T. (1887). *Ber. dtsch. chem. Ges.* **20**, 1279.
Perkin, A. G. & Steven, A. B. (1903). *J. chem. Soc.* **83**, 192.
Ross, H. (1911). *Die Pflanzengallen*. Jena: Gustav Fischer.
—— (1916). *Die Pflanzengallen Bayerns*. Jena: Gustav Fischer.
Willstätter, R. & Heiss, H. (1923). *Liebigs Ann.* **433**, 17.
—— & Stoll, A. (1918). *Liebigs Ann.* **416**, 46.

Tissue Nucleic Acids

2. THE ISOLATION AND PROPERTIES OF LIVER RIBONUCLEIC ACID

By J. N. DAVIDSON and CHARITY WAYMOUTH, *Biochemical Laboratory, Physiology Department, University of Aberdeen*

(*Received* 2 *August* 1944)

Both ribonucleic acid and desoxyribonucleic acid have recently been shown to be present in the tissues of the sheep (Davidson & Waymouth, 1944*a*), and the current view is that the ribonucleic acid is in general a cytoplasmic constituent (Mirsky, 1943; Pollister & Mirsky, 1944), while desoxyribonucleic acid is confined to the nucleus.

So far, although the presence of pentose nucleic acids has been demonstrated in many biological materials, only three such acids have been isolated and characterized. The first to be isolated was yeast ribonucleic acid, whose structure has been reviewed by Gulland (1938, 1944). In addition to *d*-ribofuranose it contains small amounts of *l*-lyxose (Gulland, Barker & Jordan, 1943; Gulland & Barker, 1943) and is usually considered to be composed of tetranucleotide units in which the bases are adenine, guanine, uracil and cytosine in equimolecular proportions. The pancreas pentose nucleic acid was considered by Jorpes (1928, 1934) to be probably a pentanucleotide containing three molecules of purine (guanine:adenine ratio = 2:1) and two of pyrimidine. Jorpes (1924) isolated the crystalline brucine salts of cytidine and uridine phosphoric acids from hydrolysates of this nucleic acid, and concluded that the pyrimidines were cytosine and uracil.

The third pentose nucleic acid to be characterized was isolated from tobacco mosaic virus (Loring, 1939; Cohen & Stanley, 1942). It appears to be very similar to yeast ribonucleic acid and on hydrolysis has yielded guanine, adenine, cytosine and the brucine salt of an acid similar to, and probably isomeric with, yeast uridylic acid (Loring, 1939).

Liver tissue contains a desoxyribonucleic acid very similar to thymus desoxyribonucleic acid (Levene, 1922; Peters, 1911; Ishiyama, 1928; Greenstein & Jenrette, 1940). This nucleic acid has been prepared from isolated liver nuclei by Dounce (1943). The cytoplasm of liver cells contains particles of at least two sizes (Claude, 1941, 1943*a*, *b*; Bensley, 1942) composed of phospholipin nucleoprotein complexes containing a pentose nucleic acid which has not yet been isolated and characterized.

It has previously been shown that sheep liver is a tissue with a relatively high content of pentose nucleic acid (Davidson & Waymouth, 1944*a*). From a dry ethanol-ether powder of sheep liver the nucleic acids were extracted with 10% sodium chloride and precipitated as lanthanum salts. The precipitate was rich in pentose. Moreover, the sodium chloride extract contained a substrate for the enzyme ribonuclease, and the amount of this substrate, in terms of phosphorus, indicated a ribonucleic-acid content of the same order as that found by the pentose estimations. The evidence for the presence of a pentose nucleic acid, presumably a ribonucleic acid, in liver is thus fairly conclusive (Davidson & Waymouth, 1944*b*), but it has seemed desirable that this nucleic acid should be isolated and characterized.

METHODS

Principle. For the extraction of the nucleic acids mild methods are essential and the use of alkali has been avoided. The methods usually employed for the preparation of desoxyribonucleic acid are sufficiently drastic to break down much of the ribonucleic acid. Extraction with 10% NaCl as used by Clarke & Schryver (1917) for the preparation of yeast ribonucleic acid has proved satisfactory provided that the liver proteins were first denatured with ethanol. Extraction of fresh minced liver with 10% NaCl gave tissue extracts from which separation of the nucleic acids was difficult.

From the 10% NaCl extract, the nucleic acids were precipitated with ethanol. The crude pentose nucleic acid was purified through the barium salt by the method of Jorpes (1934) and was finally precipitated from glacial acetic acid.

Procedure. Fresh sheep liver was minced, mixed well with 3 vol. of ethanol, and allowed to stand overnight. The ethanol was sucked off on a large Buchner funnel and the tissue suspended in two further successive portions of ethanol. After being washed with ether, it was dried in air and ground to a fine powder in a mill. 200 g. portions of this powder were suspended in 800 ml. 10% NaCl and allowed to stand overnight in the refrigerator. The suspension was then filtered through muslin and the solid residue mixed with 400 ml. 10% NaCl, heated for 30 min. in a boiling water-bath, and cooled. The mixture was again filtered through muslin and the residue washed with a further 200 ml. 10% NaCl at room temperature and filtered. The filtrates were combined (1000 ml.) and centrifuged. A small amount of precipitate and of floating fatty material was discarded. To the slightly cloudy fluid 2 vol. of ethanol were slowly added with brisk stirring, and the mixture allowed to stand overnight in the refrigerator. As much as possible of the supernatant fluid was then siphoned off and the precipitate centrifuged down, washed once with 70% ethanol, twice with absolute ethanol, dried in a vacuum desiccator, and powdered. Yield 4–5 g.

4 g. of this crude nucleic acid were ground in a mortar with 60 ml. water and centrifuged. The residue was ground with 40 ml. water and centrifuged. To the combined supernatant fluids, 0·25 vol. 20% barium acetate (pH 6·8) was added and the precipitate of barium salts centrifuged down and washed three times with 5% barium acetate. The precipitate was then ground in an ice-cold mortar with 4–5 ml. ice-cold N-HCl and centrifuged. The precipitate was washed with successive 3 ml. portions of ice water until the washings were free from Ba^{++} ions, and ice-cold N-NaOH added dropwise until the solution was no longer acid. Alkaline reaction was carefully avoided. The solution was made up to about 10 ml. with ice-water and made just acid with acetic acid. A small precipitate was centrifuged down and discarded. The supernatant fluid was poured into 10 vol. glacial acetic acid with constant stirring. The precipitate was centrifuged down, washed three times with ethanol, once with ether and dried *in vacuo*. Yield 200–250 mg.

500 mg. of this material were cautiously dissolved in water with the addition of the minimum amount of ice-cold alkali. A small amount of material remained undissolved. The solution (10 ml.) was made just acid with acetic acid and centrifuged. The supernatant fluid was poured into 10 vol. glacial acetic acid, washed twice with ethanol, once with ether, and dried *in vacuo*. Yield 300 mg.

Properties of the nucleic acid

The liver ribonucleic acid prepared in this way is a light brown powder, slightly soluble in water and giving an acid solution; it is also easily soluble in dilute alkali. It gave a negative biuret test and negative tests for desoxypentoses. Tests for pentoses were strongly positive. After drying at 100° it gave N = 15·0–15·1, P = 6·7–7·6%. Both these values are lower than the theoretical values which, for a tetranucleotide of the yeast ribonucleic acid type, $C_{38}H_{47}O_{28}N_{15}P_4$, are N = 16·3, P = 9·5%. It has been pointed out that the figures for P in many samples of nucleic acid tend to be low (Fletcher, Gulland, Jordan & Dibben, 1944; Fletcher, Gulland & Jordan, 1944). A sample of yeast ribonucleic acid precipitated twice from glacial acetic acid gave N = 15·0, P = 7·7%.

The purine content was determined by hydrolyzing a sample of the nucleic acid in 1·2N-H_2SO_4 at 100° for 1 hr. The purines were precipitated with copper hydroxide and copper bisulphite as described by Kerr & Blish (1932) and Kerr (1940), and the purine N determined. It varied in different samples from 55·1 to 61·3% of the total N. For a tetranucleotide of the yeast nucleic acid type the figure would be 66·7%, for a pentanucleotide with three purine nucleotides 75%, and for a hexanucleotide with four purine nucleotides 80%.

The easily hydrolyzable P, i.e. that derived from the purine nucleotides, was determined by hydrolyzing a portion of the nucleic acid for 2½ hr. with 5% (v/v) H_2SO_4 at 100°. Inorganic P was then determined by the method of Allen (1940). It amounted to 54·2–54·7% of the total P. For a tetranucleotide of the yeast ribonucleic acid type the figure is 53% (Jorpes, 1934), for a pentanucleotide 63%, for a hexanucleotide 69%.

The pentose content of the nucleic acid was estimated by the method of Mejbaum (1939), but with 30 min. heating as recommended by Schlenk (1942). The greenish blue colour was examined in the Hilger Spekker absorptiometer and the readings plotted against phosphorus content. The results obtained with two different samples of the liver ribonucleic acid are shown in Fig. 1. The points obtained for the two samples, and the points for yeast ribonucleic acid, lie on the same line. Liver ribonucleic acid and yeast ribonucleic acid therefore contain the same amounts of pentose relative to phosphorus.

The action of crystalline ribonuclease (Kunitz, 1940) on liver ribonucleic acid was also examined. To each of three conical 15 ml. centrifuge tubes containing 1·5 ml. of a 0·1% solution of the sodium salt of liver ribonucleic acid, 2·4 ml. veronal acetate buffer pH 6·12 were added. To one tube 0·01 mg. crystalline ribonuclease was added. To one of the remaining two (control) tubes 4·0 ml. 0·25% uranyl

acetate in 2·5% trichloroacetic acid were added immediately. The other control tube and the tube containing enzyme were incubated at 37° for 1 hr. before receiving the uranyl acetate reagent. The precipitate produced by the reagent was centrifuged down, washed with 2 ml. 0·125% uranyl acetate

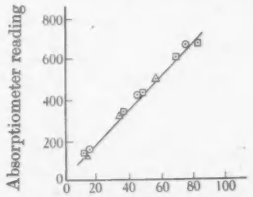


Fig. 1. Relationship between the amounts of pentose (absorptiometer reading—colorimetric method of Mejbaum, 1939) and of phosphorus found in different amounts of specimens of ribonucleic acids. △—△ Liver ribonucleic acid. ⊙—⊙ Liver ribonucleic acid. ⊡—⊡ Yeast ribonucleic acid.

in 1·25% trichloroacetic acid, dissolved in 0·5M-Na_2CO_3, and transferred to a digestion flask for total P estimation. The P content of the precipitate from the tube containing the enzyme was 39% of that of the precipitate from the control tubes, i.e. 61% of the total P has been rendered non-precipitable by the uranyl acetate reagent. A similar figure was obtained with yeast ribonucleic acid.

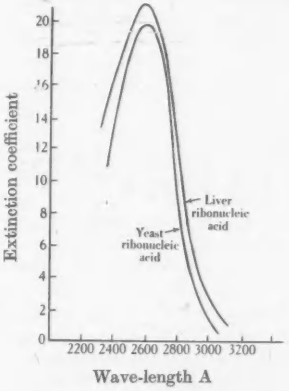


Fig. 2. Absorption spectrum of liver ribonucleic acid and yeast ribonucleic acid as sodium salts in 0·005M-phosphate buffer pH 7·2. Concentration = 0·0023% ribonucleic acid.

Absorption spectrum. The absorption spectrum of the nucleic acid was measured in a Hilger medium quartz spectrograph. We are indebted to Dr A. Clow for carrying out these measurements.

The nucleic acid was dissolved in the minimum amount of dilute NaOH and diluted to a suitable concentration (40–160 μg. P/100 ml.) with 0·005M-phosphate buffer pH 7·2. A sample of yeast ribonucleic acid (British Drug Houses Ltd.) was examined at the same time under the same conditions. The curves are shown in Fig. 2. The curve for yeast ribonucleic acid shows the typical absorption maximum in the region of 2600 A, and is similar to the curves for pentose nucleic acids shown in the papers of Caspersson (1936) (yeast and pancreas pentose nucleic acids), Lavin, Thompson & Dubos (1938) (pneumococcal pentose nucleic acid) and Lavin, Loring & Stanley (1939) (virus pentose nucleic acid). The liver ribonucleic acid gives a curve very similar to that from yeast ribonucleic acid, the maximum being even slightly higher than with the latter.

Products of hydrolysis. The products of hydrolysis of the nucleic acid were investigated, the procedure of Bredereck & Richter (1938) as used by Gulland & Barker (1943) being followed. The hydrolysis followed the same course when the process was carried out with 1 g. yeast ribonucleic acid, and 1 g. of liver nucleic acid.

Liver ribonucleic acid (1 g.) was heated with 10 ml. 2% (w/v) H_2SO_4 for 2 hr. in a flask attached to a reflux condenser and immersed in an oil-bath at 105–110°. The mixture was transferred to a centrifuge tube and the concentration of H_2SO_4 increased to 3·8% (w/v) by the addition of conc. H_2SO_4. The tube was allowed to stand overnight at room temperature. The precipitate of guanine sulphate was centrifuged down and washed with 1 ml. of 2% H_2SO_4.

To the supernatant fluid and washings $Ba(OH)_2$ solution was added until the reaction was just neutral. The $BaSO_4$ was centrifuged down and washed with a few drops of water. The supernatant and washings were taken down to 5 ml. in a shallow dish in a vacuum desiccator over conc. H_2SO_4. Solid matter which separated out was centrifuged down, washed with a few drops of water and discarded, and the supernatant fluid and washings were taken down to dryness in the vacuum desiccator. The dry residue was rubbed up with 0·5 ml. water and transferred to a centrifuge tube. Insoluble matter was centrifuged down, washed with 2 drops of water and discarded. To the supernatant and washings in a fresh centrifuge tube cooled in ice, 7 ml. pyridine were added with stirring. The cytidylic acid fraction was centrifuged down and washed twice with 1 ml. pyridine.

The supernatant and washings were taken to dryness in a vacuum desiccator over $CaCl_2$. The residue was dissolved in 2 ml. water and ethanol added until no further precipitate appeared (about 5 ml.). The precipitate was centrifuged down. The supernatant fluid was taken to dryness in a vacuum

desiccator over $CaCl_2$ and dissolved in 2·5 ml. water. A small insoluble residue was discarded. 0·5 ml. of a 2% ethanolic brucine solution was added and the tube set aside for 5 days at room temperature. The brucine salts of the uridylic acid fraction were centrifuged down.

The supernatant fluid was made alkaline with hot saturated $Ba(OH)_2$. The precipitate was discarded and the supernatant was extracted three times with chloroform to remove brucine. The aqueous layer was then centrifuged to remove a small amount of insoluble material and was exactly neutralized with 10% H_2SO_4. The $BaSO_4$ was centrifuged down and washed with a few drops of water. In the combined supernatant fluid and washings pentose was determined by the method of Mejbaum (1939). The amount present was 29·1 mg., calculated as ribose.

To 15 mg. of the pentose, dried from the frozen state in a centrifuge tube, 20 mg. *p*-bromophenylhydrazine were added, followed by 0·48 ml. water and 0·1 ml. 50% acetic acid. The mixture was allowed to stand overnight at room temperature. The yellow precipitate of the *p*-bromophenylhydrazone was centrifuged down, washed with a little absolute ethanol and recrystallized from 50% ethanol. It softened at 168° and melted at 169°. A mixture of this material with ribose *p*-bromophenylhydrazone made from pure *d*(−)-ribose (prepared from guanosine by the method of Levene & Clark (1921)) softened at 168° and melted at 169–170°. The *p*-bromophenylhydrazone made from this sample of ribose softened at 169° and melted at 171–172°. The *p*-bromophenylhydrazone made from the pentose of yeast ribonucleic acid melted at 166°. Arabinose *p*-bromophenylhydrazone prepared in exactly the same way melted at 154–155°.

The guanine sulphate precipitate was stirred with hot 10% (w/v) H_2SO_4 until no more would dissolve, and was centrifuged while hot. A moderately large insoluble residue was discarded. To the hot solution excess ammonium hydroxide solution was added. The precipitate of free guanine was centrifuged down and washed with a little dilute ammonia.

The supernatant and washings were boiled to remove ammonia, cooled and treated with saturated picric acid solution. On standing in the refrigerator overnight, a precipitate of adenine picrate appeared. It was centrifuged down and recrystallized from 25% (v/v) acetic acid.

The amounts of purine and pyrimidine bases obtained have not been sufficient for the complete characterization of all the bases.

DISCUSSION

The composition of the material under examination, the pentose content, the absence of positive tests for proteins and desoxypentose, indicate that it is a nucleic acid of the pentose nucleic acid type. Since the pentose appears to be ribose the acid can be correctly designated 'liver ribonucleic acid'.

The presence of ribose in liver tissue was suggested by Levene & Jacobs (1909), who maintained that guanylic acid from liver contained *d*-ribose: Winter (1927) identified ribose derivatives in a mixture of goat liver and muscle.

It has been generally assumed that the sugar in pentose nucleic acids and nucleotides is *d*-ribose but the evidence for this has until recently not been altogether satisfactory. Gulland & Barker (1943), using the benziminazole method (cf. Moore & Link, 1940; Dimler & Link, 1943), have now demonstrated conclusively that the sugar in yeast nucleic acid and its related nucleotides is *d*-ribose. This method of pentose identification is probably superior to any other, but it requires a larger amount of sugar than has been available to us in the present investigation. The *p*-bromophenylhydrazone method requires much less pentose and has been used by Schlenk (1942) to show that the sugar in cozymase is *d*-ribose. The melting-points for ribose-*p*-bromophenylhydrazone quoted in the literature (cf. Levene & Jacobs, 1909) vary widely. In the present instance, however, the agreement between the melting-points of the *p*-bromophenylhydrazones prepared at the same time under the same conditions from ribose and the pentose of the liver nucleic acid and recrystallized from the same solvent, together with the results of the mixed melting-point, is sufficient to justify the conclusion that the pentose is ribose. The melting-point of the *p*-bromophenylhydrazone of arabinose is lower than that of ribose, and that of the xylose derivative is lower still. The possibility of the presence of small amounts of *l*-lyxose in the liver ribonucleic acid such as are present in yeast ribonucleic acid (Gulland & Barker, 1943) cannot be excluded, but the amounts of nucleic acid available have been too small for any attempt to be made to detect it.

The similarity between liver ribonucleic acid and yeast ribonucleic acid is revealed by the pentose content, by the absorption spectrum, by the action of ribonuclease, and by the nature of the products of hydrolysis. Moreover, the purine content and the easily hydrolyzable P content suggest that the liver ribonucleic acid, like yeast ribonucleic acid, is composed of tetranucleotide units containing equimolecular amounts of purines and pyrimidines. It is possible, therefore, that liver ribonucleic acid may differ from the pentose polynucleotide of the pancreas, which may be a pentanucleotide containing three purine nucleotide residues (Jorpes, 1934).

SUMMARY

1. A method for the isolation of a ribonucleic acid from liver tissue is described.

2. The nucleic acid is free from protein and from desoxypentose. It resembles yeast ribonucleic acid in its pentose content, in its absorption spectrum, in its action as a substrate for ribonuclease and in its content of purine and easily hydrolyzable phosphorus. Among the hydrolysis products *d*-ribose has been identified as the *p*-bromophenylhydrazone.

We should like to thank Dr Neil Campbell of Edinburgh University for supplying us with a specimen of pure *p*-bromophenylhydrazine, and for his advice on the preparation of hydrazones. This work was carried out during the tenure of a Carnegie Teaching Fellowship by one of us (J.N.D.). A grant for scientific assistance from the Medical Research Council and an expenses grant from the Carnegie Trust for the Universities of Scotland to one of us (J.N.D.) are gratefully acknowledged.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bensley, R. R. (1942). *Science*, **96**, 389.
Bredereck, H. & Richter, C. (1938). *Ber. dtsch. chem. Ges.* **71** (B), 718.
Caspersson, T. (1936). *Skand. Arch. Physiol.* **74**, Suppl. no. 8.
Clarke, G. & Schryver, S. B. (1917). *Biochem. J.* **11**, 319.
Claude, A. (1941). *Cold Spr. Harb. Symp. Quant. Biol.* **9**, 263.
—— (1943*a*). *Science*, **97**, 451.
—— (1943*b*). *Biol. Symp.* **10**, 111.
Cohen, S. S. & Stanley, W. M. (1942). *J. biol. Chem.* **144**, 589.
Davidson, J. N. & Waymouth, C. (1944*a*). *Biochem. J.* **38**, 39.
—— —— (1944*b*). *Nature, Lond.*, **154**, 207.
Dimler, R. J. & Link, K. P. (1943). *J. biol. Chem.* **150**, 345.
Dounce, A. L. (1943). *J. biol. Chem.* **147**, 685.
Fletcher, W. E., Gulland, J. M. & Jordan, D. O. (1944). *J. chem. Soc.* p. 33.
—— —— —— & Dibben, H. E. (1944). *J. chem. Soc.* p. 30.
Greenstein, J. P. & Jenrette, W. V. (1940). *J. nat. Cancer Inst.* **1**, 91.
Gulland, J. M. (1938). *J. chem. Soc.* p. 1722.
—— (1944). *J. chem. Soc.* p. 208.
—— & Barker, G. R. (1943). *J. chem. Soc.* p. 625.
Gulland, J. M., Barker, G. R. & Jordan, D. O. (1943). *Nature, Lond.*, **153**, 20.
Ishiyama, N. (1928). *Hoppe-Seyl. Z.* **178**, 217.
Jorpes, E. (1924). *Biochem. Z.* **151**, 227.
—— (1928). *Act. med. scand.* **68**, 253, 503.
—— (1934). *Biochem. J.* **28**, 2102.
Kerr, S. E. (1940). *J. biol. Chem.* **132**, 147.
—— & Blish, M. E. (1932). *J. biol. Chem.* **98**, 193.
Kunitz, M. (1940). *J. gen. Physiol.* **25**, 15.
Lavin, G. I., Loring, H. S. & Stanley, W. M. (1939). *J. biol. Chem.* **150**, 259.
—— Thompson, R. H. S. & Dubos, R. J. (1938). *J. biol. Chem.* **125**, 75.
Levene, P. A. (1922). *J. biol. Chem.* **53**, 441.
—— & Clark, E. P. (1921). *J. biol. Chem.* **46**, 19.
—— & Jacobs, W. A. (1909). *Ber. dtsch. chem. Ges.* **42**, 2102, 2469, 2474, 2703.
Loring, H. S. (1939). *J. biol. Chem.* **130**, 251.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Mirsky, A. E. (1943). *Advances in Enzymology*, **3**, 1.
Moore, S. & Link, K. P. (1940). *J. biol. Chem.* **133**, 293.
Peters, A. W. (1911). *J. biol. Chem.* **10**, 373.
Pollister, A. W. & Mirsky, A. E. (1944). *Nature, Lond.*, **153**, 711.
Schlenk, F. (1942). *J. biol. Chem.* **146**, 619.
Winter, L. B. (1927). *Biochem. J.* **21**, 467.

Tissue Nucleic Acids

3. THE NUCLEIC ACID AND NUCLEOTIDE CONTENT OF LIVER TISSUE

By J. N. DAVIDSON and CHARITY WAYMOUTH, *Biochemical Laboratory, Physiology Department, University of Aberdeen*

(*Received* 2 *August* 1944)

It has been shown previously (Davidson & Waymouth, 1944 *a*, *d*) that ribonucleic acids are widespread in animal tissues and are present in high concentrations in rapidly growing embryonic tissues, although the proportion of ribonucleic acid to desoxyribonucleic acid appears in most cases to be similar in the embryo and the corresponding adult tissue. A ribonucleic acid has been isolated from the liver of the sheep and its properties described (Davidson & Waymouth, 1944*b*). It has been demonstrated histochemically in the cytoplasm of the liver cell (Davidson & Waymouth, 1944*c*). It is of some interest, therefore, to determine the amount of this nucleic acid present in liver tissue and its possible variation under different circumstances. For such an examination liver tissue has many advantages. It is possible to compare normal adult liver not only with the embryonic organ but with the regenerating organ and with liver tumours produced by the administration of such agents as dimethylaminoazobenzene (butter yellow). This fact has already been utilized by Burk (1942) in an

examination of the glycolysis mechanisms in liver in different states. He was able to show that only in malignant liver tissue was anaerobic glycolysis markedly increased (see also Dickens & Weil-Malherbe, 1943). In regenerating liver it was normal, and the high values obtained for embryonic liver could be to a large extent accounted for by the amount of erythropoietic tissue present at this stage (Burk, 1942; Norris, Blanchard & Povolny, 1942). When the nucleic acid contents of embryonic and adult livers are compared, this difference in cellular composition must be recognized.

Higher nucleic acid contents have been found in embryonic sheep and fowl livers than in the adult tissues (Davidson & Waymouth, 1944*a*) and in newborn lamb than in adult sheep (Robertson & Dawbarn, 1929), and a fall in liver nucleic acid has been noted during embryonic development in the rabbit (Masing, 1911) and in the rat (Dumm, 1943).

Variations in the total nucleic acid contents of adult livers have also been observed with the age and nutritional state of the animal (Rondoni, 1941). A rise in nucleic acid concentration on fasting has been observed in the livers of many species (Kossel, 1882; Tichmeneff, 1914; Cahn & Bonot, 1928; Rosenthal & Drabkin, 1943; Kosterlitz & Cramb, 1943; Kosterlitz, 1944 *a*, *b*), though this is accompanied by a fall in liver weight, due to decrease in cell volume, so that there is in fact a fall in liver nucleic acid (as well as in phospholipin and in protein) relative to the original body weight (Kosterlitz & Cramb, 1943).

When the median and left lateral lobes (65–75 % of the whole organ) of the liver of the rat are removed, the remaining lobes undergo a process of rapid growth until in 28 days the liver has been restored to its original weight (Higgins & Anderson, 1931). This growth process is usually termed 'regeneration', although Sulkin (1943) points out that it would more correctly be designated a compensatory hyperplasia. The most active phase occurs about the third day after operation when mitotic figures are most numerous (Burk, 1942; Norris, Blanchard & Povolny, 1942). During the first 24 hr. after partial hepatectomy the mass of the residual liver fragment increases markedly by increase in cell size without cell division (Brues, Drury & Brues, 1936). During the process of regeneration the nucleic acid metabolism of the liver is profoundly affected. Using P^{32}, Brues, Tracy & Cohn (1942) have shown that while in normal rat liver the (desoxyribo)nucleic acid P has a lower turnover rate than the non-(desoxyribo)nucleic acid protein-bound P, in the regenerating liver the turnover rate of the nucleic acid P is increased up to the level of the non-(desoxyribo)nucleic acid protein-bound P.

The liver is, moreover, an organ in which experimental tumours can be produced without great difficulty by feeding the dyestuff 'butter yellow', *p*-dimethylaminoazobenzene (cf. Sugiura & Rhoads, 1941). The process involves a 'proliferation of connective tissue cells in the portal systems with degeneration of the contiguous liver cells, a typical regenerative proliferation of bile duct and liver epithelium leading ultimately to non-architectural nodular hyperplasia and a macroscopically hob-nailed liver' in which three types of tumour may arise, liver-cell carcinoma, bile-duct carcinoma and bile-duct cystadenoma (Orr, 1940). Liver is indeed one of the few tissues in which it is possible to make a direct comparison between tumour tissue and its tissue of origin (Dickens & Weil-Malherbe, 1943). Tumour formation is associated with a rise in nucleic acid content (Fujiwara, Nakahara & Kishi, 1937; Dickens & Weil-Malherbe, 1943).

With rat liver, therefore, it is possible to compare normal adult liver tissue with rapidly growing liver tissue in the case of the embryonic or 'regenerating' organ and with the cirrhotic or tumour-bearing organ.

METHODS

Animals. All experiments were carried out on adult hooded rats supplied by the Rowett Institute and fed, unless otherwise stated, on the stock diet described by Thomson (1936).

Pregnant animals were used 1–2 days before parturition was expected, in order that embryos of reasonable size might be obtained. The embryos were removed from the uterus, quickly weighed, and the livers excised, pooled and dealt with as for adult liver.

In the experiments involving regenerating liver, rats under 'Nembutal' anaesthesia were partially hepatectomized by the method of Higgins & Anderson (1931). For the first 16 hr. after operation the animals received only a solution of glucose in water. Subsequently they received stock diet. In a control group of animals the abdomen was opened under 'Nembutal' anaesthesia and closed again without removal of hepatic tissue. The rats were killed on the third day after operation, after a 24 hr. fast.

For the experiments on hepatic tumours three groups of rats were chosen. Two groups were fed on husked but unpolished rice, to each kg. of which were added 25 ml. of a 3 % solution of dimethylaminoazobenzene (A.R.—British Drug Houses Ltd.) in arachis oil. Each rat received a slice of raw carrot daily and water *ad lib.*, and was starved for 24 hr. before use. One group was used after 100 days and another after 150 days. The third group of rats received rice with arachis oil but without dye, and served as controls. Their livers were examined at the end of 100 days.

Estimation of nucleic acids, etc. The animals were killed by decapitation and the livers immediately excised and divided into portions thus: One portion was ground in ice-cold trichloroacetic acid for estimation of nucleotide and nucleoside by the method of Kerr (1940). A second portion was dried in a tared beaker at 100° to constant weight for determination of water content. A third portion was fixed for histological examination. A fourth small portion was used for determination of total nucleoprotein phosphorus (NPP) or residual P by the method of Berenblum, Chain & Heatley (1939). The remainder of the tissue was used for

the estimation of the relative amounts of the two types of nucleic acid.

The tissue was minced, dehydrated with several successive portions of ethanol and of ether, and dried. The dried material was transferred to a stoppered 50 ml. centrifuge tube and shaken for four 1 hr. periods with successive portions of about 40 ml. 0·1 N-HCl. This process removes acid-soluble P, including simple nucleotides. The residue was washed twice with ethanol and was then extracted for two successive 2 hr. periods at 65° under a reflux condenser with an ethanol-chloroform mixture (3:1). It was then washed with ether, dried, ground to a fine powder in a mechanical mortar and passed through a sieve (120 mesh). In cases where the amount of powder available from one liver was small, material from several livers was pooled for analysis. Total P was estimated in the extracted powder by a modification of the method of Allen (1940).

Ribonucleic acid phosphorus and desoxyribonucleic acid phosphorus. The relative amounts of ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) in the extracted powder were determined by two different methods. One involved the extraction of the nucleic acids with 10% NaCl, their precipitation as lanthanum salts, and analysis of the precipitate for pentose and desoxypentose by modifications of methods previously described (Davidson & Waymouth, 1944*a*). As this procedure in the case of liver tissue involved certain losses which were relatively greater for desoxyribonucleic acid than for ribonucleic acid, direct estimations of pentose and desoxypentose were also undertaken on the extracted tissue powders. Such estimations yield useful information in the case of liver tissue though it is doubtful if they could be applied to other tissues indiscriminately.

(*a*) *Direct method.* Portions of powder expected to contain 100–200 μg. RNAP were used for pentose, and 100–300 μg. DNAP for desoxypentose, estimations.

Pentose estimation. Appropriate portions of powder (usually 20 mg.) were weighed into each of two graduated 10 ml. centrifuge tubes. To one was added 2 ml. water and 2 ml. Mejbaum's (1939) reagent (100 mg. orcinol in 10 ml. conc. HCl containing 0·1% $FeCl_3.6H_2O$), and to the other 2 ml. water and 2 ml. of HCl containing 0·1% $FeCl_3.6H_2O$ but no orcinol. Both tubes were heated with stirring in a boiling water-bath for exactly 10 min. and cooled. The blue-green material was extracted successively with 2, 2 and 1·5 ml. amyl alcohol. The amyl-alcohol layers were separated by centrifuging, combined, mixed with 1 ml. ethanol, and made up to a total volume of 8 ml. with amyl alcohol. Exactly similar extractions were carried out on the blank tube (without orcinol). The coloured solution was read against the blank in the Hilger Spekker absorptiometer, with the Blue (no. 7) colour filter. A calibration curve was prepared from yeast ribonucleic acid, for which 2 ml. portions at various concentrations (25–200 μg. P) were treated as in the estimation.

Estimation of desoxypentose was carried out by a modification of the diphenylamine reaction employed by Sevag, Smolens & Lackman (1940) and Dische (1930). An appropriate amount of powder (usually 200 mg.) was heated in a graduated 10 ml. centrifuge tube with 8 ml. 0·1 N-HCl for 20 min., with frequent stirring. After cooling, the volume was made up to 8 ml. and the mixture was centrifuged. A 3 ml. portion was heated for exactly 6 min. in a briskly boiling water-bath with 8 ml. diphenylamine reagent (1 g. A.R. diphenylamine, twice recrystallized from ethanol, 2 ml. conc. H_2SO_4 and 98 ml. A.R. glacial acetic acid). A blank was prepared from a second 3 ml. of the supernatant fluid and 8 ml. acetic acid. The cooled solution was read against the blank in the Hilger Spekker absorptiometer, with the Red (no. 1) colour filter. A calibration curve was prepared from desoxyribonucleic acid, 4 ml. portions being hydrolyzed in HCl of final concentration 0·1 N, 3 ml. of the supernatant being used for the development of the colour with 8 ml. of reagent.

(*b*) *Method for lanthanum salts.* The nucleic acids were extracted from the tissue powders with 10% NaCl and precipitated as lanthanum salts. The solution of nucleic acids recovered from the lanthanum precipitate was then used for the estimation of pentose and desoxypentose by a modification of the method previously employed (Davidson & Waymouth, 1944*a*).

For the nucleic acid extraction, a suitable quantity of tissue powder (e.g. 400 mg.) was weighed into a 15 ml. conical centrifuge tube and 4 drops triacetin added. Extraction with 10% NaCl was carried out with five successive portions: (*a*) 3 ml. overnight at 0°, (*b*) 2 ml. for 30 min. at 100°, (*c*) 2 ml. for 30 min. at 100°, (*d*) 2 ml. for 10 min. at 100°, and (*e*) 2 ml. for 10 min. at 100°, respectively.

The extracts were combined and made up to 10 ml. 0·5 ml. was taken for total P estimation. 9 ml. were pipetted into a 25 ml. centrifuge tube and 1 ml. 2% lanthanum acetate and 10 ml. ethanol added. After 1 hr. at 0° the precipitate was centrifuged down and washed twice with 3 ml. 0·2% lanthanum acetate. The moist precipitate was decomposed with two successive 0·25 ml. portions of 0·5 M-Na_2CO_3. The lanthanum carbonate was centrifuged down and the extract made up to 5 ml.

The extract was treated as follows: (i) 0·5 ml. was taken for total P estimation. (ii) 0·2–0·3 ml. was pipetted into a pyrex tube for estimation of pentose by a modification of the method of Mejbaum (1939). The volume was made up to 2 ml. with water and 2 ml. Mejbaum's reagent (*vide supra*) were added. The tube was heated in a boiling water-bath for 30 min. as recommended by Schlenk (1942) and was then cooled. 2 ml. ethanol and 4 ml. water were added and the green colour read off in the Hilger Spekker absorptiometer (Filter no. 7) with a calibration curve drawn up in terms of RNAP and made from liver ribonucleic acid precipitated as lanthanum salt and treated as described above. (iii) 3 ml. were taken for the colorimetric estimation of desoxypentose by the diphenylamine reaction, and were pipetted into a conical graduated centrifuge tube containing 1 ml. 0·55 N-HCl. The tube was heated in a boiling water-bath for 20 min., cooled, and the volume made up to 4 ml. 8 ml. freshly made diphenylamine reagent (*vide supra*) were added and the tube was placed in the briskly boiling water-bath for 6 min., quickly cooled, and the blue colour read off after 5 min. in the Hilger Spekker absorptiometer (Filter no. 1), with a calibration curve drawn up in terms of desoxyribonucleic acid phosphorus (DNAP) and made from thymus desoxyribonucleic acid precipitated as lanthanum salt.

Neither the total P of the extracted powder nor the ratio RNAP/DNAP was appreciably altered when the fresh livers were allowed to stand overnight at room temperature.

RESULTS

The results of the nucleoprotein phosphorus (NPP) and moisture estimations are shown in Table 1. The moisture content in most cases remains constant at about 70% but is higher in embryo liver and in liver

Table 1. *Water and nucleoprotein phosphorus* (NPP) *content of rat livers*

Mean values and the standard error are shown in each case. The figures in brackets are the numbers of animals employed.

Type of liver tissue	Water content (%)	Nucleoprotein phosphorus (NPP) (mg./100 g.) Wet wt.	Nucleoprotein phosphorus (NPP) (mg./100 g.) Dry wt.	Weight of rats (g.)	Weight of livers (g.)	Weight of liver as % body wt.
Normal male (fed)	69·9±0·412 (9)	134± 2·56 (14)	449± 7·33 (14)	361±16·09 (9)	10·6±0·55 (6)	2·89±0·10 (6)
Normal male (fasted 24 hr.)	69·7±0·195 (11)	152± 2·65 (16)	502± 9·84 (16)	316±22·83 (8)	7·9±0·54 (8)	2·51±0·10 (8)
Normal female (fed)	70·0±0·263 (12)	137± 3·74 (12)	452± 13·25 (12)	172± 5·49 (11)	7·1±0·36 (7)	4·17±0·15 (7)
Normal female (fasted 24 hr.)	69·9±0·237 (12)	142± 3·66 (12)	473± 12·81 (12)	168± 6·80 (10)	5·1±0·22 (6)	3·39±0·13 (6)
Pregnant female (fasted 24 hr.)	71·0±0·258 (6)	156± 3·64 (6)	543± 12·06 (6)	—	—	—
Embryo	80·6±0·271 (8)	174±13·27 (8)	908±101·5 (8)	2·6±0·24 (8)	—	—
Regenerating (male)	70·0±0·395 (9)	153± 3·18 (9)	512± 12·04 (9)	350±15·12 (8)	7·3±0·32* (8)	—
Laparotomy (male)	69·5±0·229 (6)	150± 1·64 (8)	490± 5·47 (8)	353±12·04 (6)	9·0±0·40 (7)	—
Control diet (male)	70·6±0·111 (6)	149± 2·44 (6)	505± 6·97 (6)	224± 3·05 (6)	—	—
Liver tumours (100 days) (male)	73·8±0·444 (6)	143± 1·64 (6)	543± 10·95 (6)	179± 6·27 (6)	—	—
Liver tumours (150 days) (male)	76·0±0·731 (5)	128± 6·54 (5)	535± 40·74 (5)	163± 4·95 (4)	—	—

* The mean weight of liver tissue removed 3 days previously at partial hepatectomy was 6·8±0·46 g.

tumour. The high moisture content of the rapidly growing tissues of embryos and tumours has already been commented on (Davidson & Waymouth, 1944*a*). The NPP figures for the rat liver are slightly higher than those quoted by other authors (Rondoni, 1941; Berenblum, Chain & Heatley, 1939; Kosterlitz & Cramb, 1943) and are closer to those of Fujiwara, Nakahara & Kishi (1937). The NPP rises on fasting. In our series of normal male rats (340 ± 60 g.) the difference between the NPP of livers of fed and starved animals is statistically significant. In the series of female rats (170 ± 30 g.) it is not significant but the difference between the total P content of the extracted powders from the livers of fed and starved female rats is significant. The weight of the liver as a percentage of the body weight shows a significant fall in the starved animal (male or female). The liver of a pregnant rat has a particularly high NPP content but the highest values are found in the embryo. The weights of the embryos used varied from 3·4 to 1·5 g. and the NPP values from 125 to 246 mg./100 g. wet weight or 596 to 1440 mg./100 g. dry weight.

The regenerating livers show slightly higher NPP values than those of the controls, but the difference is not statistically significant. On the other hand, the difference between the values for the 100-day liver tumours and those of the controls is significant.

The NPP of the tumours is lower, on a wet-weight basis, than that of the livers of rats on the control diet, but on a dry-weight basis it is higher, on account of the high water content of the tumours.

Results of the nucleotide, etc. estimations are shown in Table 2. There is no significant difference in nucleotide concentration or in total soluble purine concentration in the livers of fed and fasted rats. This confirms the tentative conclusion arrived at by Rapoport, Lever & Guest (1943). Embryo liver, like other embryonic tissues, has a particularly low nucleotide concentration. The nucleotide and total purine concentration in regenerating livers is significantly higher than that of controls. In the 100-day tumours the nucleotide concentration is significantly lower on a wet-weight, but not on a dry-weight, basis than that of the controls. No appreciable variations in the nucleoside + free purine fraction were found.

The results of the analyses of the extracted tissue powders are shown in Table 3. The total P content runs parallel with the NPP figures but is expressed here in terms of dry fat-free material. In most cases more than 90 % of this total P can be accounted for in terms of ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP). The powders from livers of starved animals show a lower RNAP content and a higher DNAP content

Table 2. *Nucleotide N, nucleoside N, and free purine N contents of rat livers*

The mean and the standard error are shown in each case. The number of animals is given in brackets.

Type of liver tissue	Nitrogen content* (mg./100 g.) Nucleotide N	Nucleoside N + free purine N	Total acid-soluble purine N
Normal male (fed)	30·6±1·01 (6)	9·7±1·20 (6)	40·3±0·62 (6)
Normal male (fasted 24 hr.)	31·1±0·68 (10)	10·2±0·74 (9)	41·4±0·83 (9)
Embryo	17·1±0·89 (6)	7·3±0·30 (6)	24·4±0·79 (6)
Regenerating	36·1±1·46 (8)	10·9±1·41 (8)	47·0±1·87 (8)
Laparotomy	32·0±1·09 (8)	9·9±0·59 (7)	41·1±1·18 (7)
Control diet	31·4±1·48 (6)	9·9±0·71 (6)	44·0±1·23 (6)
Tumours (100 days)	28·8±0·28 (6)	10·7±0·67 (6)	39·6±0·92 (6)
Tumours (150 days)	17·3±3·31 (5)	9·9±1·24 (5)	27·3±3·43 (5)

* The sum of nucleotide N + nucleoside N + free purine N gives the total acid-soluble purine N content.

Table 3. *Ribonucleic acid phosphorus* (RNAP) *and desoxyribonucleic acid phosphorus* (DNAP) *content of extracted liver powders, as estimated directly and on the precipitate of lanthanum salts*

The figure in brackets shows the number of powders examined. Each powder represents, in most cases, pooled material from several animals.

Type of liver tissue	Total P in extracted powder (mg./100 g.)	RNAP as % total P: Direct	RNAP as % total P: From lanthanum ppt.	DNAP as % total P: Direct	DNAP as % total P: From lanthanum ppt.	RNAP + DNAP as % total P: Direct	RNAP + DNAP as % total P: From lanthanum ppt.	Ratio: RNAP/DNAP: Direct	Ratio: RNAP/DNAP: From lanthanum ppt.
Fed	554±10·72 (10)	75·3±2·33 (10)	81·9±1·89 (10)	17·6±0·59 (10)	10·6±0·51 (10)	92·6	93·5	4·3	8·1
Fasted	583±20·46 (9)	66·0±2·86 (9)	75·8±1·82 (9)	20·2±0·84 (9)	13·3±0·52 (9)	86·7	89·1	3·1	5·8
Pregnant	774 (1)	75	77	17·7	10	93	86	4·2	7·8
Regenerating	690 (2)	72	90	18	8·5	90	98	4·0	10·9
Laparotomy	603 (2)	85	88	22·2	10·5	107	97	3·8	8·5
Control diet	716 (1)	61	85	13·7	13	75	98	4·5	6·5
Tumours	738 (2)	71	75	19·5	18·9	90·5	94	3·6	4·0
Embryo	1720 (1)	49·5	70	33·7	28	83·5	98	1·5	2·5

than do those of fed rats, and both these differences are statistically significant. The ratio RNAP/DNAP is therefore reduced by fasting. This ratio is almost the same in the regenerating as in the control livers. In the tumours it is lower than in the corresponding controls, but the actual amounts of RNAP and DNAP determined in the tumour powders are both higher than those in the controls. Owing to the high total P content of livers of pregnant rats the total RNAP content is particularly high. In embryonic liver the ratio is low, and the extracted powder contains a higher RNAP content and DNAP content than does any of the other liver powders, but the relative amount of DNAP is higher. This is due in part at least to the presence of erythropoietic cells in the embryo liver. A lower RNAP/DNAP ratio in the embryo liver than in the adult liver has already been noted in the case of the sheep (Davidson & Waymouth, 1944*a*).

The ratios of RNAP/DNAP obtained for various forms of rat liver tissue indicate that a large proportion (some 80%) of the total nucleic acid of rat liver tissue is present in the form of ribonucleic acid. Similar ratios have been obtained with the liver tissue of other species (sheep, pig, horse, man).

The ratios obtained from the analysis of the precipitate of lanthanum salts are in most cases considerably higher than those found on direct analysis of the liver powder. Owing to losses in extraction of the nucleic acids, in precipitation as lanthanum salts and on resolution from the lanthanum precipitate, the amount of P in the material recovered from the lanthanum precipitate is, in the case of liver tissue, only about 60% of that in the tissue powder. These losses are proportionately greater for desoxyribo- than for ribonucleic acid, and the RNAP/DNAP ratios are therefore high. Nevertheless the ratios obtained for various forms of liver show the same relative variations among themselves as do the ratios obtained by direct analysis.

DISCUSSION

The rise in NPP concentration in liver tissue found in fasting animals confirms the earlier observations of other workers (Kossel, 1882; Tichmeneff, 1914; Cahn & Bonot, 1928; Rosenthal & Drabkin, 1943; Kosterlitz & Cramb, 1943). Kosterlitz (1944*a*, *b*) has shown that rats starved or placed on a protein-poor diet show a loss in stainable material and of

mitochondria in the cytoplasm of the liver cell. The particulate components of the cytoplasm are reported by Claude (1943*a*, *b*), Bensley (1942), Hoerr (1943) and Lazarow (1943) to contain a nucleic acid of the pentose type which is presumably identical with the ribonucleic acid which we have isolated from liver tissue (Davidson & Waymouth, 1944*b*). The fall which we have observed in the RNAP/DNAP ratio in fasting rats would be consistent therefore with a diminution in the phospholipin-ribonucleoprotein complex content of the liver cytoplasm. The loss on fasting of liver weight as a percentage of body weight is in agreement with the results of other workers (e.g. Kosterlitz & Cramb, 1943).

Comparison of normal adult liver tissue with rapidly growing liver tissue from the embryo is rendered difficult by the peculiar cellular content of the latter but such a comparison can be made between normal and regenerating liver. The livers of rats submitted to laparotomy give figures for nucleic acid and nucleotide content which are very close to those obtained for normal male starved rats. The regenerating livers differ appreciably only in one respect, their high simple nucleotide and total soluble purine concentration. In this they differ from other rapidly growing tissues (embryonic and neoplastic), which have characteristically low acid-soluble nucleotide concentrations (Davidson & Waymouth, 1944*a*, *d*). Such low concentrations are found in the embryonic rat liver and in the liver tumours.

The NPP of the 100-day liver tumours is higher than that of the control livers when the comparison is made on a dry-weight basis, but the difference is due largely to the increased water content of the tumours. In no case was the difference between the NPP (dry-weight basis) of the liver tumours and that of normal rat liver as great as that recorded by Fujiwara, Nakahara & Kishi (1937). On a wet-weight basis the NPP figures are of the same order for liver tumours, livers from rats on the control diet and livers from normal fasting male rats. On the other hand, the P contents of the extracted liver powders are much higher in the case of both liver tumours and livers from rats on the control diet than in the case of livers of normal starved rats. The P content of the extracted powder from liver of pregnant rats is also at the same high level.

Caspersson, Nyström & Santesson (1941) and Caspersson & Santesson (1942) have reported the presence of high concentrations of ribopolynucleotides in the cytoplasm of some tumour cells. Although the concentration of RNAP in the liver tumours is higher than that of the controls the difference is in no case very great. The DNAP concentration is also raised and the RNAP/DNAP ratio is lower than that of the controls.

It is now clear that liver tissue contains ribonucleic acid, that this acid is found in the cytoplasm of the liver cell, that it forms a large proportion of the total nucleic acid of liver tissue, and that it may vary independently of the nuclear desoxyribonucleic acid.

SUMMARY

1. The nucleic acid and nucleotide concentrations have been examined in a series of livers from fed and starved rats, from the pregnant and embryo rat, from the regenerating rat liver and in liver tumours induced by feeding butter yellow.

2. The total nucleic acid concentration, as measured by the nucleoprotein phosphorus, rises on fasting, is high in pregnancy and very high in the embryo. It is not appreciably altered in the regenerating liver, and is slightly raised in liver tumours. The acid-soluble nucleotide concentration is unaltered on fasting, is raised in regenerating livers, is low in liver tumours and very low in embryo livers.

3. Of the total nucleic acid a large proportion (70–90 %) is present as ribonucleic acid. On fasting, the ribonucleic acid concentration falls by about 12 % while the desoxyribonucleic acid concentration rises, both changes being statistically significant. The ratio of ribonucleic acid P to desoxyribonucleic acid P therefore falls on fasting, is the same in regenerating as in control livers, and is lower in liver tumours and in embryo livers than in the normal liver, although in both liver tumour and in the embryo, particularly the latter, the concentrations of both ribonucleic acid and desoxyribonucleic acid are higher than in normal liver.

This work was carried out during the tenure of a Carnegie Teaching Fellowship by one of us (J.N.D.). A grant for scientific assistance from the Medical Research Council and an expenses grant from the Carnegie Trust for the Universities of Scotland to one of us (J.N.D.) are gratefully acknowledged. We wish to thank Mr E. Alexander for valuable technical assistance.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bensley, R. R. (1942). *Science*, **96**, 389.
Berenblum, I., Chain, E. & Heatley, N. G. (1939). *Biochem. J.* **33**, 68.
Brues, A. M., Drury, D. R. & Brues, M. C. (1936). *Arch. Path.* **22**, 658.
Brues, A. M., Tracy, M. M. & Cohn, W. E. (1942). *Science*, **95**, 558.
Burk, D. (1942). *A Symposium on Respiratory Enzymes*, p. 235. Chicago: University of Chicago Press.
Cahn, T. & Bonot, A. (1928). *Ann. Physiol. Physicochim. biol.* **4**, 399, 485, 781.

Caspersson, T., Nyström, C. & Santesson, L. (1941). *Naturwissenschaften*, **29**, 29.
—— & Santesson, L. (1942). *Acta radiol., Stockh.*, Suppl. 46.
Claude, A. (1943*a*). *Science*, **97**, 451.
—— (1943*b*). *Biol. Symp.* **10**, 111.
Davidson, J. N. & Waymouth, C. (1944*a*). *Biochem. J.* **38**, 39.
—— —— (1944*b*). *Biochem. J.* **38**, 375.
—— —— (1944*c*). *Proc. Roy. Soc. Edin.* **62** B, 96.
—— —— (1944*d*). *Nutr. Abstr. Rev.* **14**, 1.
Dickens, F. & Weil-Malherbe, H. (1943). *Cancer Res.* **3**, 73.
Dische, Z. (1930). *Mikrochemie*, **8**, 4.
Dumm, M. E. (1943). *J. cell. comp. Physiol.* **21**, 27.
Fujiwara, T., Nakahara, W. & Kishi, S. (1937). *Gann*, **31**, 51.
Higgins, G. M. & Anderson, R. M. (1931). *Arch. Path.* **12**, 186.
Hoerr, N. L. (1943). *Biol. Symp.* **10**, 185.
Kerr, S. E. (1940). *J. biol. Chem.* **132**, 147.
Kossel, A. (1882). *Hoppe-Seyl. Z.* **7**, 7.
Kosterlitz, H. W. (1944*a*). *Biochem. J.* **38**, *Proc.* xiv.
—— (1944*b*). *Nature, Lond.*, **154**, 207.
—— & Cramb, I. D. (1943). *J. Physiol.* **102**, 18*P*.
Lazarow, A. (1943). *Biol. Symp.* **10**, 9.
Masing, E. (1911). *Arch. exp. Path. Pharmak.* **66**, 71.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Norris, J. L., Blanchard, J. & Povolny, C. (1942). *Arch. Path.* **34**, 208.
Orr, J. W. (1940). *J. Path. Bact.* **50**, 393.
Rapoport, S., Lever, E. & Guest, G. M. (1943). *J. biol. Chem.* **149**, 57.
Robertson, T. B. & Dawbarn, M. C. (1929). *Aust. J. exp. Biol. med. Sci.* **6**, 261.
Rondoni, P. (1941). *Schweiz. med. Wschr.* **71**, 1354.
Rosenthal, O. & Drabkin, D. L. (1943). *J. biol. Chem.* **150**, 131.
Schlenk, F. (1942). *J. biol. Chem.* **146**, 619.
Sevag, M. G., Smolens, J. & Lackman, D. B. (1940). *J. biol. Chem.* **134**, 523.
Sugiura, K. & Rhoads, C. P. (1941). *Cancer Res.* **1**, 3.
Sulkin, N. M. (1943). *Anat. Rec.* **73**, 107.
Thomson, W. (1936). *J. Hyg., Camb.*, **36**, 24.
Tichmeneff, N. (1914). *Biochem. Z.* **59**, 326.

The Determination of Thiocyanate in Blood Serum

By R. G. BOWLER, *Clinical Laboratory and Medical Unit, The London Hospital*

(*Received* 31 *July* 1944)

Body water is distributed in three main compartments, blood plasma, cell fluid and the fluid in the intercellular spaces (interstitial fluid). In order to study changes in the distribution of water between these compartments it is necessary to be able to measure their volumes accurately. Crandall & Anderson (1934) have described a method for determining the volume of water available to thiocyanate. It has been inferred that this volume is equivalent to the interstitial fluid volume (Peters, 1942). Their method involves the injection of a known amount of sodium thiocyanate intravenously followed by the determination of its concentration in the blood serum after sufficient time has been allowed for its complete distribution.

The concentration of thiocyanate in the serum is determined by treating a trichloroacetic acid filtrate of serum with a solution of ferric nitrate; the orange colour which develops is measured colorimetrically. The standard is prepared by adding a known amount of thiocyanate to serum and treating it by the same process.

In this laboratory, attempts to follow changes in the volume of the interstitial fluid in cases of traumatic shock have met with little success. Determinations at intervals on the same patient gave inconsistent results. The technique of estimation was investigated, and it was found that when the colour was compared photoelectrically with that of an aqueous solution of NaSCN instead of a standard filtrate, recoveries were about 7·5% low. This was due to the trichloroacetic acid interfering with the colour development in the filtrate. Attempts to obviate this error by the use of different protein precipitants (*p*-toluenesulphonic acid, uranyl nitrate, etc.) were unsuccessful; low recoveries were still obtained.

Conditions affecting the colour development were then studied. It was found that in Crandall & Anderson's method the concentration of the reagent is far below the optimum. The use of a modified method gave quantitative recoveries which were unaffected by the trichloroacetic acid.

EXPERIMENTAL

Colorimeter. A tungsten filament source was used, infrared radiation being removed by a 2 mm. thickness of Chance's 'Calorex' glass. The solution was measured in a 2 cm. solution thickness. A blue filter was used, Ilford 'Spectrum Blue' no. 602, with a maximum transmission at 4700 A.

Standard thiocyanate solution. When 500 mg. NaSCN have been injected into an average-sized man, the concentration of thiocyanate in the serum is about 3 mg. NaSCN/100 ml. A solution of NaSCN slightly stronger than 0·1 N was made by dissolving 11·5 g. of the salt in water and diluting to 1 l. This was standardized by titration against 0·1 N-$AgNO_3$. The concentration was then adjusted to be exactly 0·1 N-NaSCN, equivalent to 8·11 g. NaSCN/l. By diluting 35·1 ml. of the 0·1 N-solution to 1 l., and again diluting 35·1 ml. of this solution to 1 l., a concentration of

0·01 mg. NaSCN/ml. was obtained. 3 ml. of this solution contained about the same amount of NaSCN as there is in 1 ml. of serum.

The effect of ferric nitrate concentration on colour intensity. To a series of tubes, each containing 0·03 mg. NaSCN and 2·5 ml. 2 N-HNO_3, varying amounts of ferric nitrate were added. The volume was made up to 10 ml. A set of controls was similarly prepared, the thiocyanate solution being replaced by an equal volume of water. The amount of ferric nitrate added varied from 3 g. to 0·05 mg., or 60,000 to 1 equivalents of the thiocyanate. The colour intensities of the solutions and controls were measured, soon after development, against distilled water. The extinction due to the thiocyanate was obtained by deducting that of the control from the solution. The results are shown in Fig. 1.

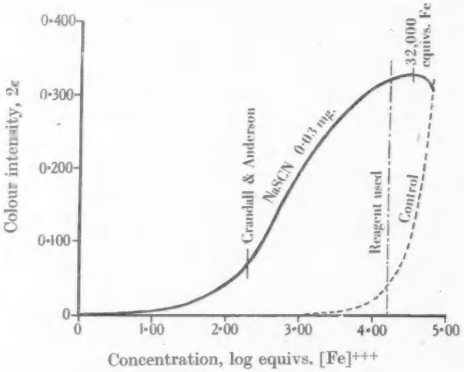


Fig. 1. Effect of ferric nitrate concentration on the colour intensity of the thiocyanate-ferric nitrate complex.

It is seen that a very large excess of ferric nitrate was required to produce maximum colour. In this high concentration, partial hydrolysis caused a large increase in the blank colour. For optimum conditions it is necessary to use a concentration which, whilst giving the maximum colour intensity for the thiocyanate, will have a small blank value; 0·8 g. (16,000 equivalents) is suitable. The amount of thiocyanate used in this experiment was twice that which will usually be present in a serum filtrate. The ratio, which will be in the optimum range, will therefore be of the order of 32,000 equivalents of $[Fe]^{+++}$ to 1 equivalent of $[SCN]^{-}$. Interfering substances should then have a minimum effect on the colour development.

The effect of acid concentration on the colour intensity. A series of tubes was prepared each containing 0·03 mg. NaSCN, 1·5 g. ferric nitrate (30,000 equivalents) and varying amounts of a standardized solution of nitric acid. The volume was made up to 10 ml. in each case. The final concentration of acid ranged from 0 to 1·13 N. Control samples were also prepared, differing only in that they contained no thiocyanate. The colour intensities of the solutions and controls were measured against distilled water. The extinction due to the thiocyanate was obtained by deducting the control value. The results are shown in Fig. 2.

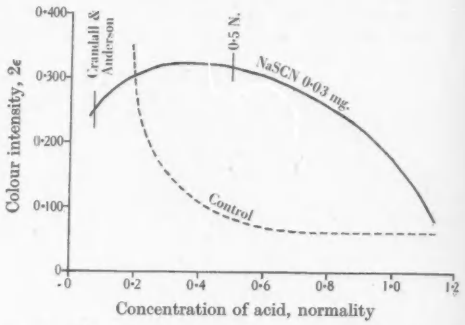


Fig. 2. Effect of acid concentration on the colour intensity of the thiocyanate-ferric nitrate complex.

Maximum colour was developed in 0·35 N-acid, but the blank value was high, because of hydrolysis of the ferric nitrate. In 0·5 N-acid the blank was reasonably low and the colour intensity due to thiocyanate was still nearly maximal.

Stability of the coloured complex. Solutions were prepared containing 0·03 mg. NaSCN in 5 ml. of water; 0·8 g. ferric nitrate in 5 ml. of N-HNO_3 was added in artificial light, and the colour intensity measured at once against controls. The solutions were then allowed to stand in different light intensities, some in a dark cupboard, others in electric light (about 4 ft. below a 60 W. lamp), the remainder in dull daylight. Measurements were made at intervals. Results are shown in Fig. 3.

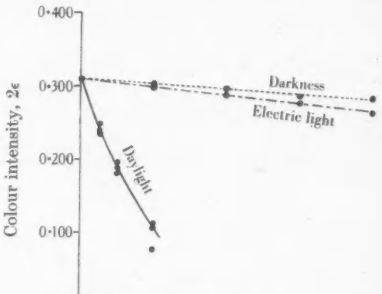


Fig. 3. Stability of the coloured complex of thiocyanate with ferric nitrate.

Exposure of the coloured solutions to light caused them to fade. There was a decrease in the colour intensity of about 20 %/hr. in daylight, 1 %/hr. in artificial light and 0·5 %/hr. when kept in the dark up to 16 hr.

The reagent should be added in artificial light and the colour intensity measured within 15 min. There will then be no appreciable error due to fading.

Summary of the conditions necessary for optimum colour development

(1) Ferric nitrate should be present in large excess, in the ratio of at least 27,000/1 g., or 16,000/1 equivalent, $Fe(NO_3)_3.9H_2O$/NaSCN.

(2) The final solution should be 0·5N-HNO_3.

(3) Exposure of the coloured solution to daylight must be carefully avoided.

(4) The colour intensity should be measured within 15 min. after the addition of the reagent.

THE ESTIMATION OF THIOCYANATES IN BLOOD SERUM

Method

Reagents

Trichloroacetic acid solution. 20 g. trichloroacetic acid are dissolved in 100 ml. of water and filtered.

Ferric nitrate-nitric acid reagent. 80 g. $Fe(NO_3)_3.9H_2O$ are dissolved in 250 ml. 2N-HNO_3, made to 500 ml. with water and filtered.

Estimation

Oxalated or citrated plasma must not be used because of the colour which the anticoagulant produces with ferric nitrate.

1 ml. of serum is measured into a test-tube, 6·5 ml. water and 2·5 ml. trichloroacetic acid solution added, mixed by inversion several times, allowed to stand for about 10 min. and filtered through a Whatman no. 40 paper 9 cm. diameter. 5 ml. of the filtrate are treated with 5 ml. of the ferric nitrate reagent in absence of daylight, mixed and measured within 15 min. against the control.

1 ml. of a sample of serum withdrawn before injection of thiocyanate is taken through the same process for a control.

Results

Standard curve

Various amounts of the standard thiocyanate solution were diluted to 5 ml. with water, 5 ml. of the ferric nitrate reagent added, and the colour intensity measured against a control of 5 ml. reagent diluted with 5 ml. of water.

It is seen from Table 1 that the Beer-Lambert law is obeyed. The colour produced is about 50% greater than that given by Crandall & Anderson's method.

Table 1. *Proportionality of colour produced by thiocyanate and ferric nitrate*

NaSCN (mg.)	Colour intensity (extinction, 2 cm.)
0·030	0·312
0·025	0·263
0·020	0·209
0·015	0·157
0·010	0·105
0·005	0·053

Recoveries

Known amounts of the standard solution of thiocyanate were added to normal sera; they were then estimated as described above and the colour intensity compared with the standard curve. In a series of 25 sera the mean recovery was 100%, the maximum deviation from the mean 3%, and the standard deviation 0·8. Under these conditions the trichloroacetic acid does not interfere with the colour development.

Nature of the coloured complex

Considerable use has been made of the reaction between ferric salts and thiocyanates in the colorimetric determination of small amounts of iron (Snell & Snell, 1936). According to Schlesinger & Van Valkenburgh (1931), the colour in aqueous solution is due to the ion $[Fe(CNS)_6]^{'''}$ when there is an excess of thiocyanate present. In the determination of thiocyanate by the addition of a large excess of ferric salt, as described in this paper, the coloured complex appears to be different in nature. A complete study of these unstable complexes could not be undertaken at the time, but it is of interest to note the results of some preliminary investigations.

On shaking the aqueous solution with a non-polar solvent like ether or benzene, the complex formed when thiocyanate was in excess was completely extracted from the aqueous phase. The complex formed when a large excess of iron was present was insoluble in the non-polar solvent. This suggests that the first substance is weakly ionized and the second strongly ionized in the aqueous solution.

Electrophoresis of the aqueous solution caused the brown colour to move towards the cathode when an excess of iron was present. Schlesinger & Van Valkenburgh (1931) found that the excess-thiocyanate complex migrated towards the anode. It would appear that the colour is associated with the positive ion in the first case and with the negative ion in the latter.

Absorption curves in the visual region, measured with a Hilger-Nutting spectrophotometer, showed that in both cases the maximum absorption is in the violet. A peak was observed at about 4550A when the excess iron complex was examined. A satisfactory curve was not obtained when excess thiocyanate was used, owing to rapid fading. Further investigation is necessary before the nature of the complex can be found.

SUMMARY

1. Conditions affecting the colorimetric determination of small amounts of thiocyanate in blood serum by the ferric thiocyanate reaction have been studied.

2. Trichloroacetic acid used for the removal of protein may interfere with colour development when Crandall & Anderson's method is used.

3. By increasing the concentrations of ferric nitrate and nitric acid in the reagent to optimum conditions, quantitative results were obtained.

4. A photoelectric method for the determination of thiocyanate in blood serum which gives accurate results is described.

I wish to thank Dr A. C. Crooke and Dr C. J. O. R. Morris for encouragement and advice. The expenses of this research were defrayed from the Yarrow Fund of the London Hospital.

REFERENCES

Crandall, L. A., Jr. & Anderson, M. X. (1934). *Amer. J. digest. Dis. Nutr.* **1**, 126.

Peters, J. P. (1942). *Ann. Rev. Physiol.* **4**, 93.

Schlesinger, H. L. & Van Valkenburgh, H. B. (1931). *J. Amer. chem. Soc.* **53**, 1212.

Snell, F. D. & Snell, C. T. (1936). *Colorimetric Methods of Analysis*. New York: D. Van Nostrand Co. Inc.

A New Method for the Oxidation of Aneurin to Thiochrome and a Procedure for the Determination of Aneurin in Oats

By W. I. M. HOLMAN, *The Rowett Institute, Aberdeen*

(*Received* 28 *July* 1944)

It was desired as part of an investigation on the composition of oats to determine the aneurin content of a large number of samples with sufficient accuracy to distinguish relatively small differences. The methods of Hennessy & Cerecedo (1939) and Harris & Wang (1941) did not give in our hands the required degree of precision. The method of Nicholls, Booth, Kent-Jones, Amos & Ward (1942) for flour is not applicable to oats since the sample is not digested with a proteolytic enzyme before extraction and only free aneurin is determined. It was considered advisable to study in some detail the oxidation of aneurin to thiochrome, the extraction of aneurin from oats and the purification of extracts by the use of a selective adsorbent.

Existing methods for the determination of aneurin by oxidation to thiochrome are modifications of the method of Jansen (1936). Various workers have drawn attention to factors likely to lead to error in the application of this method, viz. ferricyanide concentration (Nicholls *et al.* 1942); partition between aqueous and *iso*butanol layers (Herd, Mundy & Ridyard, 1943); instability of *iso*butanol solutions of thiochrome in ultra-violet (u.v.) light (Nicholls *et al.* 1942; Wokes, Organ, Still & Jacoby, 1944).

EXPERIMENTAL

Oxidation of aneurin to thiochrome

In a search for an oxidizing agent weaker than ferricyanide, which might render the oxidation less difficult to control and the extraction with *iso*butanol unnecessary, a number of metal oxides used as oxygen electrodes was examined. Such oxides provide a small but constant partial pressure of oxygen. Heating an alkaline solution of aneurin with HgO for 10 min. at 100° was found to be the most promising, but the presence of solid HgO caused erratic results, apparently because of adsorption of thiochrome on the HgO. A saturated solution of HgO in 25% (w/v) KCl solution which is alkaline in reaction gave not only reproducible results but also a much greater yield of thiochrome, as indicated by the intensity of its fluorescence after heating for 10 min. at 100°.

A more satisfactory method of preparing such a solution is by mixing solutions of $HgCl_2$, KCl (or NaCl) and KOH (or NaOH):

$$HgCl_2 + 2KOH \rightleftharpoons HgO + 2KCl + H_2O.$$

Precipitation of mercuric oxychloride does not occur if the proportion of $HgCl_2$ is sufficiently small. When 5 ml. of 25% (w/v) KCl solution were used the maximum amount of 1% $HgCl_2$ solution which could be added without risk of precipitation of mercuric oxychloride when the solution was subsequently made alkaline was found to be 1 ml. To 5 ml. of a freshly prepared solution of aneurin in 25% KCl was added 1 ml. of 1% $HgCl_2$ solution followed by 4 ml. of NaOH solution. A fluorescence developed which, when the solution was heated, soon reached its maximum intensity. The rate of development increased as the temperature was raised, but destruction of thiochrome occurred at high temperatures. At 40°, the most suitable temperature, the fluorescence reached its maximum intensity in 15 min., and destruction did not occur on further heating. The concentration of alkali used affected the development of the fluorescence slightly, but the temperature of the solution when the alkali was added had a greater effect. The maxi-

mum intensity of fluorescence obtainable by suitable heat treatment after the addition of the alkali varied according to the temperature of the solution when the alkali was added. The highest result was obtained by adding the alkali at 0° and then heating the solution for 15 min. at 40°.

It was found necessary always to mix the $HgCl_2$ completely with the KCl solution before adding the alkali, otherwise mercuric oxychloride was precipitated and did not subsequently dissolve, leading to low and erratic results. It was also found essential for the solution of aneurin in 25% (w/v) KCl to be neutral or acid in reaction and for the alkali to be added after the $HgCl_2$. Otherwise the development of the fluorescence was almost entirely suppressed. A faint opalescence appeared when the alkali was added and the solution heated, but it could be dispersed by addition of an equal volume of acetone, which also increased the intensity of fluorescence. A higher proportion of acetone caused precipitation of KCl.

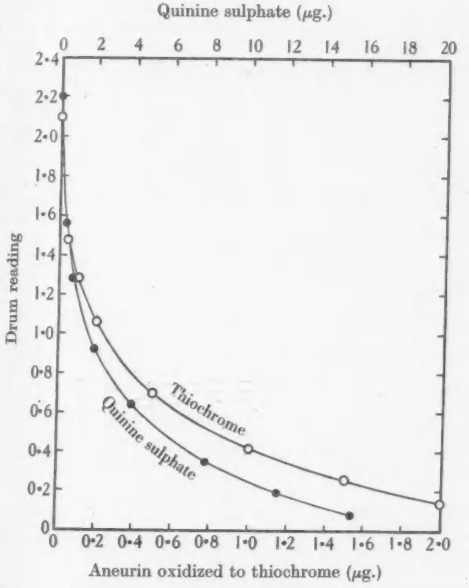


Fig. 1. Calibration curves for thiochrome and quinine sulphate using the Spekker fluorimeter.

When suitable conditions were adopted, highly reproducible results were obtained for the oxidation of 0–2 μg. aneurin. The fluorescence of the solution in u.v. light was matched in a Spekker fluorimeter against an equal volume of a standard solution of quinine sulphate in 0·1 N-H_2SO_4 containing 1 μg./ml., with a violet filter (no. 7) in front of the right photocell. The aneurin content could be obtained from the drum reading by reference to a calibration curve prepared with known amounts of pure aneurin. Such a curve is shown in Fig. 1. It is apparent that the drum reading does not decrease proportionately as the amount of aneurin increases. That this lack of proportionality does not arise from a variation in the proportion of thiochrome produced from different amounts of aneurin is indicated by the close similarity between the curve for aneurin and that for quinine sulphate, which is also included in the figure. It should be noted that the drum reading of the fluorimeter decreases as the intensity of fluorescence increases.

The results tabulated in Table 1 illustrate the effects of varying the experimental conditions on the values obtained as indicated by the drum readings. Except where otherwise stated, 4 ml. of 0·06 N-NaOH were added at room temperature, the fluorescence was developed by heating for 15 min. at 40° and, on cooling, 10 ml. of acetone were added. The tabulated results furnished a guide for establishing the procedure outlined on p. 394. It may be noted that with the present procedure the intensity of fluorescence decreases very gradually with prolonged exposure to u.v. light, the loss during 5 min. being equivalent to only about 3% of the thiochrome. The band of u.v. light passes through only a small part of the solution and deterioration is confined to that region. It has been found that if the solution is mixed before each reading, at least four successive readings, each involving a 15 sec. exposure, may be taken without any detectable diminution of the fluorescence.

The use of Decalso for the adsorption of aneurin

The method described above was applicable only to pure solutions of aneurin. Extracts of oats contain extraneous materials which fluoresce in u.v. light, as well as substances which interfere with the oxidation to thiochrome. The bulk of these impurities must be removed before the oxidation to thiochrome is performed. Several adsorbents were examined for this purpose, viz. Decalso, Superfiltrol and Lloyd's reagent. Decalso, although a much weaker adsorbent than Lloyd's reagent or Superfiltrol, was found to be much more selective, and has the further advantage that adsorbed aneurin may be readily eluted with 25% KCl solution. Lloyd's reagent and Superfiltrol adsorbed a greater proportion of impurities along with the aneurin, and aneurin could only be eluted by an alkaline solution. In such cases it would therefore be necessary to oxidize to thiochrome in the presence of the adsorbent. Jowett's (1940) technique was tested in preference to that of Hennessy & Cerecedo (1939). It requires the use of much smaller amounts of adsorbent and in consequence the adsorption of impurities is greatly reduced. The procedure is more rapid and is capable of being more closely standardized.

The effect of various factors on the adsorption of aneurin by powdered Decalso was therefore studied using either Walpole's acetate buffers diluted from 0·2 to 0·05 M or Clark & Lubs's 0·05 M-phthalate buffers. The technique employed was as follows:

The solution containing aneurin was mixed with powdered Decalso for 1 min. and, after centrifuging, the supernatant liquid was poured off. When two successive adsorptions were necessary this liquid was transferred to a second tube containing a further lot of Decalso. After mixing and centrifuging, the supernatant liquid was rejected. The residual Decalso in the second tube was transferred with 10 ml. of water to the first tube and the total contents of the tube well mixed. After centrifuging, the supernatant liquid was siphoned off to avoid risk of stirring up the Decalso, which disperses when the salt concentration is reduced. The residue was extracted for several minutes in a boiling water-bath with 10 and then 5 ml. of 25% (w/v) KCl solution and the combined extracts were made up to 15 ml. with KCl solution and mixed; 5 ml. were taken for oxidation to

Table 1. *Effect of various factors on the development of the fluorescence*

Amount of aneurin = 1 μg.

1. Concentration of acetone	ml. acetone added*	2	4	6	8	9	10		
	Drum reading†	0·782	0·656	0·564	0·507	0·473	0·452		
2. Concentration of alkali	Normality of alkali	0·02	0·03	0·04	0·05	0·06	0·075	0·10	0·15
	Drum reading†	0·519	0·463	0·448	0·443	0·444	0·446	0·448	0·450
3. Temperature of solution when alkali is added	Temp. of solution	0°	Room temp.	40°	60°				
	Drum reading†	0·413	0·448	0·491	0·568				
4. Time and temperature‡ of heating after addition of alkali at 0°	Time at 20° (min.)	4	8	12	16	20	30	40	50
	Drum reading†	0·690	0·630	0·575	0·530	0·491	0·455	0·432	0·422
	Time at 40° (min.)	2	4	8	12	16	20	30	40
	Drum reading†	0·591	0·478	0·430	0·416	0·412	0·413	0·414	0·413
	Time at 60° (min.)	1	2	4	6	10	20	30	40
	Drum reading†	0·466	0·426	0·428	0·426	0·433	0·441	0·451	0·506
5. Time and temperature‡ of heating after addition of alkali at room temp.	Time at 20° (min.)	2	4	8	12	16	20	30	40
	Drum reading†	0·505	0·498	0·488	0·478	0·468	0·461	0·455	0·452
	Time at 30° (min.)	2	4	8	12	16	20	30	40
	Drum reading†	0·492	0·479	0·468	0·460	0·453	0·452	0·448	0·450
	Time at 40° (min.)	2	4	8	12	16	20	30	40
	Drum reading†	0·492	0·462	0·449	0·448	0·449	0·448	0·450	0·448
	Time at 50° (min.)	2	4	8	12	16	20	30	40
	Drum reading†	0·450	0·448	0·453	0·452	0·450	0·450	0·449	0·449
	Time at 60° (min.)	2	4	8	12	16	20	30	40
	Drum reading†	0·448	0·448	0·447	—	0·451	0·448	0·450	0·448
	Time at 100° (min.)	1	2	3	4	5	10	25	40
	Drum reading†	0·450	0·447	0·450	0·449	0·452	0·460	0·479	0·518
6. Irradiation in ultra-violet light	Time of irradiation (min.)	0	5	10	15	20	25	30	
	Drum reading†	0·448	0·464	0·480	0·494	0·509	0·522	0·534	

* Solutions containing <10 ml. were made up with H_2O to the same final volume as that containing 10 ml.
† Of Spekker fluorimeter.
‡ The volume was corrected, after heating and cooling, for evaporation loss.

Table 2. *Effect of various factors on the adsorption of aneurin by powdered Decalso*

1. 3 μg. aneurin adsorbed from 10 ml. 0·05 M-acetate by 0·2 g. Decalso	pH of solution	3·6	4·0	4·4	4·8	5·2			
	Recovery of aneurin (%)	87	93	95	96	95			
2. 3 μg. aneurin adsorbed from 0·05 M-acetate (pH 4·8) by 0·2 g. Decalso	Volume of solution (ml.)	5	10	15	20	25			
	Recovery of aneurin (%)	96	96	96	98	98			
3. 3 μg. aneurin adsorbed from 0·05 M-acetate (pH 4·8) by two successive 0·1 g. lots of Decalso	Volume of solution (ml.)	5	10	15	20	25			
	Recovery of aneurin (%)	99	97	97	99	98			
4. 3 μg. aneurin adsorbed from 10 ml. 0·05 M-phthalate by 0·2 g. Decalso	pH of solution	2·5	3·0	3·5	4·0	4·5	5·0	5·5	6·0
	Recovery of aneurin (%)	84	90	92	93	87	81·5	74	57
5. 3 μg. aneurin adsorbed from 0·05 M-phthalate (pH 4·0) by 0·2 g. Decalso	Volume of solution (ml.)	5	10	15	20	25			
	Recovery of aneurin (%)	91	93	91	90	88			
6. 3 μg. aneurin adsorbed from 10 ml. 0·05 M-phthalate (pH 4·0) by different amounts of Decalso	Amount of Decalso (g.)	0·02	0·04	0·06	0·08	0·10	0·15	0·20	
	Recovery of aneurin (%)	73	85	91	93	93	93	93	
7. Different amounts of aneurin adsorbed from 10 ml. 0·05 M-phthalate (pH 4·0) by 0·2 g. Decalso	Amount of aneurin (μg.)	0·5	1·0	1·5	2·0	2·5	3·0	4·5	6·0
	Recovery (%)	100	96	94	95	95	93	93	92
8. 3 μg. aneurin adsorbed from 0·05 M-phthalate (pH 4·0) by two successive 0·1 g. lots of Decalso	Volume of solution (ml.)	5	10	15	20	25			
	Recovery of aneurin (%)	97	100	98	95	92			
. Different amounts of aneurin adsorbed from 10 ml. 0·05 M-phthalate (pH 4·0) by two successive 0·1 g. lots of Decalso	Amount of aneurin (μg.)	0·5	1·0	1·5	2·0	2·5	3·0	4·5	6·0
	Recovery (%)	100	100	100	100	100	100	100	100

thiochrome. Under these conditions aneurin was extracted completely from Decalso. No destruction of aneurin occurred at 100° since the extracts were always acid in reaction as a result of base exchange with the Decalso. The adsorption was not improved by raising the temperature or by increasing the time of mixing the Decalso with the solution.

When acetate buffers were used the optimum pH of adsorption was 4·8. The degree of adsorption was not affected by the volume of solution and only very slightly improved by performing two successive adsorptions with Decalso. The values obtained for the recovery of aneurin varied slightly when the experiments were repeated even though the same sample of Decalso was used. A precipitate was formed when acetone was added after the oxidation to thiochrome. This precipitate, which consisted of substances dissolved from the Decalso, was centrifuged off before the intensity of fluorescence was measured.

When phthalate buffers were used the optimum pH was 4·0. The degree of adsorption varied according to the volume of solution and the amounts of aneurin and Decalso present. No precipitate was formed when the acetone was added after the oxidation to thiochrome. Constant results were obtained when the experiments were repeated with the same sample of Decalso. The recovery of aneurin was improved considerably by performing two successive adsorptions and under certain conditions was complete.

Different samples of Decalso were found to vary in activity. Two samples purchased at different times gave 93% recovery when 0·1 g. powdered Decalso was used to adsorb 3 μg. aneurin from 10 ml. of 0·05 M-potassium acid phthalate solution, but two further samples gave recoveries of only 56·5 and 85·5%. The activity of these two samples could not be improved by preliminary purification.

The results of a series of experiments are shown in Table 2. The sample of Decalso giving 93% recovery under the conditions specified above was used. It is apparent that the conditions employed in Exp. 9 are satisfactory, but it must be noted that these results apply only when a sample of Decalso of equal or greater activity is used.

Determination of aneurin in oat extracts

Samples of oats after digestion with papain and/or takadiastase (see next section) were extracted with 0·05 M-potassium acid phthalate solution and analyzed according to the technique used in Exp. 9, Table 2. Unsatisfactory results were obtained owing to the presence of impurities. It was found that two successive adsorptions were inadequate but that after five adsorptions no further aneurin was recovered. Recovery values for added aneurin were still low, and since similar low recoveries for aneurin added to the final KCl extracts were obtained it is obvious that

Table 3. *Effect of different enzyme treatments on the extraction of aneurin from oats*

Method of extraction of aneurin	Weight* of dry matter (g.)	Aneurin added (μg.)	Aneurin found (μg.)	Aneurin content of dry matter (μg./g.)	Recovery of added aneurin (%)	Degree of proteolysis (%)
Oatmeal						
No digestion with enzymes: two extractions at 70° with 0·05 M-phthalate	1·3693	Nil	5·36	3·91	—	—
	1·3693	1·5	6·87	—	100·7	—
	1·3693	3·0	8·19	—	94·3	—
Digestion with 0·1 g. takadiastase: two cold extractions with phthalate	1·3700	Nil	6·02	4·39	—	3·1
	1·3718	1·5	7·47	—	96·0	—
	1·3688	3·0	9·00	—	99·7	—
Digestion with 0·05 g. papain and 0·1 g. takadiastase	1·3708	Nil	6·87	5·01	—	7·9
	1·3692	3·0	9·84	—	99·3	—
Digestion with 0·1 g. papain and 0·1 g. takadiastase	1·3701	Nil	7·50	5·47	—	10·4
	1·3667	3·0	10·50	—	101·0	—
Digestion with 0·15 g. papain and 0·1 g. takadiastase	1·3690	Nil	6·87	5·02	—	9·8
	1·3690	3·0	9·96	—	103·0	—
Digestion with 0·2 g. papain and 0·1 g. takadiastase	1·3637	Nil	6·57	4·82	—	9·8
Oats (with husks); variety 'Star'						
No digestion with enzymes: two extractions at 70° with 0·05 M-phthalate	1·3135	Nil	4·44	3·38	—	
	1·3148	1·5	5·97	—	101·3	
	1·3139	3·0	7·47	—	101·0	
Digestion with 0·1 g. takadiastase: two cold extractions with phthalate	1·3168	Nil	3·99	3·03	—	
	1·3162	1·5	5·55	—	104·0	
	1·3168	3·0	7·01	—	100·7	
Digestion with 0·5 g. papain and 0·1 g. takadiastase	1·3161	Nil	5·58	4·24	—	
	1·3168	3·0	8·64	—	102·0	
Digestion with 0·1 g. papain and 0·1 g. takadiastase	1·3255	Nil	5·70	4·30	—	
	1·3251	3·0	8·66	—	98·7	
Digestion with 0·15 g. papain and 0·1 g. takadiastase	1·3213	Nil	5·40	4·09	—	
	1·3223	3·0	8·27	—	95·3	
Digestion with 0·2 g. papain and 0·1 g. takadiastase	1·3273	Nil	5·40	4·07	—	

* Approximately 1·5 g. of fresh material were weighed out in every case and the exact corresponding weight of dry matter is given.

impurities adsorbed along with the aneurin and extracted by the KCl solution interfered with the oxidation to thiochrome. The interfering substances could not be removed from the oat extracts by ultrafiltration, solvent extraction or precipitation methods. The error arising from their presence could be eliminated by destroying the aneurin in part of the KCl extract with sulphite, removing SO_2 and then adding definite amounts of aneurin to samples of the solution and oxidizing to thiochrome.

4 μg. aneurin dissolved in 12 ml. of 25% (w/v) KCl solution were completely destroyed by heating for 1 hr. at 100° with 0·2 ml. of 12·5% (w/v) $Na_2SO_3.7H_2O$ solution. If the solution was acidified with HCl and SO_2 removed by aspiration there was no interference with the oxidation of aneurin added after the destruction. Oat extracts were analyzed by adsorbing the aneurin from 10 ml. by five successive 0·1 g. lots of Decalso, extracting the combined lots of Decalso with 15 ml. of 25% KCl solution and taking 3 ml. of the extract for oxidation to thiochrome in the usual manner, 2 ml. of 25% KCl solution being added before the $HgCl_2$ and NaOH solutions. The aneurin in the remaining 12 ml. of the KCl extract was destroyed by neutralizing with NaOH and heating for 1 hr. at 100° with Na_2SO_3; after removal of SO_2, the solution was made up to 12 ml., and 3 ml. portions included in a series of standards before oxidation to thiochrome. The aneurin content of the test solution was obtained by plotting the drum readings given by the three standards against the corresponding amounts of aneurin added and interpolating on the curve.

This technique is capable of giving accurate results only if there is no appreciable alteration of substances other than aneurin during the heat treatment with Na_2SO_3. The 'blank' fluorescence was unaltered when the heat treatment was extended to 2 or 3 hr., and it is therefore unlikely that it was affected by heating for 1 hr. The recovery of different amounts of aneurin added to oat extracts was complete, thus indicating that no serious change occurred in the substances which interfere with the oxidation to thiochrome.

The results of a series of recovery tests are shown in Table 3. Aneurin was added initially to weighed samples of oats before extraction. Different methods of extraction were used. The recoveries are in all cases satisfactory.

Extraction of combined aneurin from oats

The method described above was used to study the effect of different enzyme treatments on the extraction of aneurin from oats. Weighed samples of oats were heated for 5 min. at 100° with 20 ml. of 0·05 M-potassium acid phthalate solution to destroy natural enzymes. Destruction of aneurin does not occur under these conditions if sulphites are absent. Different amounts of papain and takadiastase were added to the cooled samples, which were then digested for 16 hr. at 37°. A little chloroform was added to inhibit growth of micro-organisms. After the extracts had been centrifuged their aneurin content was determined. The results, which are given in Table 3, show that considerably more aneurin is extracted after digestion with papain than when no enzyme or takadiastase alone is used and that 0·1 g. papain liberates more aneurin than 0·05 or 0·15 g. To estimate the extent to which the proteins of oats were hydrolyzed by these different treatments, the amino N content of the extracts was determined by the copper method of Pope & Stevens (1939). 'Blanks' on the enzymes were subtracted and the results, expressed as percentages of the total N in the sample, are included in Table 3. It is apparent that in all cases the proteins are hydrolyzed to only a small extent.

It is possible that further hydrolysis of the proteins may liberate more combined aneurin. An experiment was therefore conducted using 0·1 g. papain and 0·1 g. takadiastase with the addition of 1 ml. of 4% HCN to increase the proteolytic activity, the samples being digested for either 16 or 48 hr. at 37°. The effect of digesting for 16 hr. at 37° with 0·1 g. pepsin in 0·2% HCl, followed by digestion for 16 hr. at 37° with 0·1 g. takadiastase at pH 4·0, was also tested. The extracts were analyzed for aneurin and amino N. It was found necessary to dilute the extracts with 0·05 M-potassium acid phthalate solution before making the aneurin determinations, otherwise the greater amounts of impurities present caused low results to be obtained. The results, which are given in Table 4, show that although the degree of proteolysis is increased considerably, no more aneurin is extracted than when 0·1 g. papain was used without HCN. It is therefore probable that this maximum value represents the total aneurin content.

Procedure for determination of aneurin in oats

Reagents. The reagents should be prepared from 'Analar' grade chemicals and distilled water showing no fluorescence. Glassware must be scrupulously clean.

(1) 0·05 M-potassium acid phthalate solution. Dissolve 10·21 g. $COOH.C_6H_4.COOK$ in distilled water and dilute to 1 l.

(2) Papain and takadiastase. The enzyme preparations used should be tested for freedom from aneurin. Takadiastase containing either talc or lactose as a diluent may be used.

Table 4. *Effect of digestion with papain and* HCN *or pepsin on the extraction of aneurin from oatmeal*

Method of extraction of aneurin	Weight* of dry matter (g.)	Aneurin added (μg.)	Aneurin found (μg.)	Aneurin content of dry matter (μg./g.)	Recovery of added aneurin (%)	Degree of proteolysis (%)
Digestion with 0·1 g. papain, 0·1 g. takadiastase and 1 ml. of 4% HCN for 16 hr. at 37°	1·3697	Nil	7·56	5·52	—	21·6
	1·3688	3·0	10·50	—	98·3	—
Digestion with 0·1 g. papain, 0·1 g. takadiastase and 1 ml. of 4% HCN for 48 hr. at 37°	1·3697	Nil	7·56	5·52	—	29·8
	1·3682	3·0	10·44	—	96·0	—
Digestion with 0·1 g. pepsin followed by 0·1 g. takadiastase	1·3687	Nil	7·44	5·43	—	27·3
	1·3681	3·0	10·26	—	94·3	—

* See footnote to Table 3.

(3) Powdered Decalso. Grind the 60–90-mesh Decalso supplied by the Permutit Co. Ltd. in a mortar and pass through a 200-mesh sieve. Stir with distilled water and centrifuge for 30 sec. at 3000 r.p.m. Pour off the supernatant liquid and stir the residue with distilled water. Centrifuge for 30 sec. and pour off the supernatant liquid. Transfer to an evaporating basin and dry for several hours in an oven at 100°. Pass through the 200-mesh sieve to remove lumps.

Different batches of Decalso may vary in their ability to adsorb aneurin. A sample is suitable for use if 0·1 g. adsorbs over 90 % of the aneurin from a solution of 3 μg. aneurin in 10 ml. of 0·05 M-potassium acid phthalate solution.

(4) 25 % (w/v) KCl solution.

(5) 12·5 % $Na_2SO_3.7H_2O$ solution. Dissolve 2·5 g. $Na_2SO_3.7H_2O$ in distilled water by warming. Cool and dilute to 20 ml. Prepare the solution freshly before use.

(6) 1 % $HgCl_2$ solution.

(7) 0·06 N-NaOH solution. Prepare a CO_2-free solution and protect from atmospheric CO_2 by means of a soda-lime tube.

(8) Acetone. Distil from an all-glass apparatus before use. Recover acetone from residues by distillation; then re-distil from an all-glass apparatus.

(9) Standard solution of quinine sulphate. To prepare a stock solution dissolve 20 mg. quinine sulphate (dried at 100°) in 0·1 N-H_2SO_4 and dilute to 1 l. Store in the dark. To prepare a standard solution containing 1 μg./ml., dilute 5 ml. of the stock solution to 100 ml. with 0·1 N-H_2SO_4 solution.

(10) Standard solution of aneurin. Prepare a stock solution by dissolving 50 mg. pure synthetic aneurin in distilled water and diluting to 500 ml. This solution keeps satisfactorily for at least 3 months when stored in a refrigerator. To prepare a standard solution containing 1 μg./ml., dilute 1 ml. of the stock solution to 100 ml. with 25 % KCl solution. This solution must be prepared immediately before use. If results based on moisture-free aneurin are desired, determine the moisture content of the aneurin separately by drying over H_2SO_4 or at 100° and correct the results accordingly.

Procedure. Grind the sample of oats in a mill, taking care to avoid loss of any fraction. Weigh out 1·5 g. and transfer to a pyrex boiling tube. Add 20 ml. phthalate solution, mix and heat in boiling water for 5 min. with frequent mixing. Cool and add 0·1 g. papain, 0·1 g. takadiastase (both measured by volume) and 3 drops of chloroform. Stopper the tube and incubate for 16 hr. at 37°, mixing at intervals for the first few hours. Transfer to a centrifuge tube and, after centrifuging for 5 min. at 3000 r.p.m., transfer the supernatant liquid to a tube marked at the level corresponding to 30 ml. Wash the residue in the boiling tube into the centrifuge tube with 10 ml. phthalate solution and stir up the residue in the centrifuge tube. Centrifuge for 5 min. and transfer the supernatant liquid to the tube marked at 30 ml. Adjust the combined extract to pH 4·0 by adding N-HCl drop by drop and testing a micro-drop of the extract with bromocresol green. Make up to 30 ml. with phthalate solution, mix and centrifuge for 5 min.

Transfer 10 ml. of the extract to a centrifuge tube containing 0·1 g. Decalso (measured by volume). An 18 ml. round-bottomed tube is suitable. Mix well and centrifuge for 2 min. at 3000 r.p.m. Transfer the supernatant liquid to a second centrifuge tube containing 0·1 g. Decalso, mix and centrifuge for 2 min. Transfer the supernatant liquid to a third tube containing 0·1 g. Decalso and continue this process until five successive adsorptions with 0·1 g. Decalso have been made; then discard the supernatant liquid. Add 5 ml. distilled water to the first tube, washing down the sides and stirring up the Decalso and transfer the Decalso from the second tube to the first with 5 ml. distilled water. Add 5 ml. distilled water to the third tube, washing down the sides and stirring up the Decalso and transfer the Decalso from the fourth and fifth tubes to the third with 5 ml. distilled water in both cases. Centrifuge the first and third tubes for 5 min. and siphon off the supernatant liquid. Transfer the Decalso from the third tube to the first with 10 ml. of 25 % KCl solution and heat in a boiling water-bath for several minutes with frequent mixing. Cool, centrifuge for 5 min. and transfer the supernatant liquid to a pyrex boiling tube marked at levels corresponding to 12 and 15 ml. To the residue in the centrifuge tube add 5 ml. of 25 % KCl solution, heat in boiling water, cool and centrifuge for 5 min. Transfer the supernatant liquid to the tube marked at 12 and 15 ml. and make up to 15 ml. with 25 % KCl solution.

Transfer 3 ml. of the KCl extract to a boiling tube and add 2 ml. of 25 % KCl solution. This is the test solution. To prepare standards add 0·05 ml. 0·5 N-NaOH and 0·2 ml. Na_2SO_3 solution to the remaining 12 ml. of KCl extract, mix and heat in a boiling water-bath for 1 hr. Cool, acidify with 0·2 ml. N-HCl and wash down the sides with a little distilled water. Remove SO_2 by evacuating the tube while drawing air through the solution from a fine capillary. Almost neutralize the solution (0·1 ml. of 0·5 N-NaOH), checking the pH of a micro-drop with bromocresol green to make certain that the solution is still acid and make up to 12 ml. with distilled water. Transfer 3 ml. to each of three boiling tubes. Add a different amount of the standard solution of aneurin to each of the three tubes, the amounts varying over the range expected in the test solution. Then add sufficient 25 % KCl solution to bring the volume to 5 ml.

To the three standards and the test solution add 1 ml. of 1% $HgCl_2$ solution and mix completely with the KCl extract; then add 4 ml. of 0·06 N-NaOH solution with constant mixing. The temperature of the solution when the alkali is added may vary from 0 to 25° without affecting the result, provided that it is the same for test and standards. Heat in a water-bath at 40° for 15 min., cool and add 10 ml. acetone by blowing from a pipette. Transfer to the glass cell of a Spekker fluorimeter and match against the quinine sulphate standard, with a purple filter (no. 7) in front of the right photocell and two strong neutral filters in front of the left photocell. Plot the drum readings given by the three standards against the corresponding amounts of aneurin added and determine the aneurin content of the test solution by interpolation. The glass cells should always be placed with the same side in front of the photocell. The covers are not required.

The method is lengthy, but if samples are incubated overnight at 37° it is possible to complete six determinations each day.

DISCUSSION

With the method described above the recovery of aneurin added to oats was found to be $100 \pm 5\%$. Duplicates usually agree to within 2%. A higher degree of accuracy cannot be expected in view of the number of manipulations involved. The recovery values show that the adsorption technique and the oxidation to thiochrome are free from appreciable error, and although no proof has been obtained that the aneurin liberated from oats by 0·1 g. papain represents the total aneurin content, no more aneurin is extracted when the proteolytic activity is increased by means of HCN.

Existing evidence indicates that the thiochrome reaction is specific for aneurin. The possibility that the result obtained by the procedure described above includes substances other than aneurin is extremely remote, being restricted to substances capable of being destroyed by sulphite as well as of being oxidized to a fluorescent derivative.

All results have been calculated in terms of air-dry aneurin. The moisture content of the sample of aneurin used to prepare standards was 4·15%. If desired, the results may be converted to the oven-dry basis.

The method has been tested only with oats but may, with suitable modification, be applicable to other cereals and foodstuffs.

SUMMARY

1. A method is described for the oxidation of aneurin to thiochrome by means of HgO dissolved in KCl solution. The method enables pure solutions of aneurin to be analyzed rapidly and accurately, but is not directly applicable to extracts of oats.

2. An adsorption technique is described which employs powdered Decalso to remove all the aneurin from oat extracts and is sufficiently selective for the HgO method to be subsequently applied.

3. The effect of digestion with proteolytic enzymes on the extraction of combined aneurin from oats has been studied and a procedure which gives the maximum yield of aneurin is described.

The author is indebted to Mr W. Godden and Dr J. Duckworth for their helpful advice and criticism.

REFERENCES

Harris, L. J. & Wang, Y. L. (1941). *Biochem. J.* **35**, 1050.
Hennessy, D. J. & Cerecedo, L. R. (1939). *J. Amer. chem. Soc.* **61**, 179.
Herd, C. W., Mundy, L. M. & Ridyard, H. N. (1943). *Analyst*, **68**, 174.
Jansen, B. C. P. (1936). *Rec. Trav. chim. Pays-Bas*, **55**, 1046.
Jowett, M. (1940). *Biochem. J.* **34**, 1348.
Nicholls, J. R., Booth, R. G., Kent-Jones, D. W., Amos, A. J. & Ward, H. H. (1942). *Analyst*, **67**, 15.
Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
Wokes, F., Organ, J. G., Still, B. M. & Jacoby, F. C. (1944). *Analyst*, **69**, 1.

Purification and Properties of Yeast Pyrophosphatase

By K. BAILEY and E. C. WEBB, *Biochemical Laboratory, Cambridge*

(*Received* 15 *August* 1944)

An enzyme which hydrolyzes inorganic pyrophosphate to orthophosphate appears to have a wide distribution. It has been demonstrated in muscle (Lohmann, 1928); in intestinal mucosa, kidney, bone, lung and liver (Kay, 1928); in blood cells (Roche, 1931); in placenta and foetal liver (Minniti, 1939); and in various seeds (Fleury & Courtois, 1937). Bauer (1936) showed it to be present with phosphomonoesterase in autolysates of bottom yeast and obtained it free from the latter enzyme. Apart

from separations of this type, no rigorous purification has been attempted.

The pyrophosphatase from baker's yeast has now been prepared free from other phosphatases, and its specificity and kinetics studied. The best preparation, having a Q_P value of 10^5 at 38°, was essentially homogeneous electrophoretically, but attempts to crystallize it have been unsuccessful.

METHODS

Materials. Pyrophosphate: Kahlbaum, $Na_4P_2O_7.10H_2O$, free from orthophosphate; adenosine triphosphate (ATP) and K diphenylpyrophosphate preparations have been described (Bailey, 1942). Triphosphate, $Na_5P_3O_{10}.6H_2O$, was supplied by Dr A. Kleinzeller, and prepared by the method of Huber (1937); flavinadenine dinucleotide was a preparation from yeast containing 3 mg./g. and supplied by Dr A. Pirie; carnosine was prepared from horse flesh and twice recrystallized (N, found 24·6%; calc. 24·8%).

Activity estimations. The inorganic orthophosphate formed after incubating the enzyme with Na pyrophosphate in presence of Mg^{++} was estimated by the Fiske-Subbarow method. The enzyme (0·1–1 ml.) was incubated at 38° with 0·2 ml. M/10-$MgCl_2$, 0·2 ml. M/100 sodium pyrophosphate, and M/35 veronal-acetate buffer pH 7·2 (Michaelis, 1931) to a total volume of 5 ml. A series of tubes containing varying dilutions of the enzyme was always employed, and after 30 min. the samples were rinsed out into 25 ml. flasks containing the molybdate reagent, which stopped the reaction. The pyrophosphatase unit was defined as the amount of enzyme producing 1 mg. of orthophosphate P in 30 min. at 38° under the above conditions. The curve obtained by plotting orthophosphate P formed in 30 min. against enzyme concentration was linear up to 80% hydrolysis, and the activity value was calculated from this linear portion. An amount of enzyme equivalent to about 0·1 unit was required for each estimation; even in the crudest preparations the total amount of protein taken was so small that it did not interfere with the phosphate estimation. Since 1 mol. of pyrophosphate gives rise to 2 mol. of orthophosphate, the above unit corresponds in respect of turnover number to a unit based on the hydrolysis or transfer of 1 mg. P/hr. in the case of other enzymes. Activities when expressed as units/mg. protein N can be converted to Q_P values, which denote the volume in μl. of a hypothetical gas/mg. protein/hr., assuming 1 mol. of orthophosphate produced to be equivalent to 1 mol. of gas, by multiplying by the factor $22{,}400/31 \times 6$.

PURIFICATION

The chief procedures employed were fractional precipitation with $(NH_4)_2SO_4$, and fractional adsorption on calcium phosphate gel followed by elution with strong salt solution. The following summary of the main preparation describes the procedure.

1st stage. 70 lb. 'Encore' baker's yeast were minced and warmed to 38°, and mixed with 1·9 l. of toluene. After 30 min., 25 l. of tap water at 38° were added, and 18 hr. later the digest was centrifuged. The residue was mixed with 6·5 l. of water and spun down, and the semi-fluid portion, after the clear supernatant was decanted, was mixed with an equal volume of water and again centrifuged. The combined supernatants (53·3 l.) contained 1,197,000 units with an activity of 6·3 units/mg. total N.

2nd stage. The autolysate was dialyzed by trickling at a rate of 0·5 l./hr. through 15 ft. of collapsed cellophan tubing contained in a sink of running tap water. Dialysate: 58·2 l., 1,800,000 units and activity 12·2 units/mg. total N. The increase in activity is partly due to removal of dialyzable N and partly to the removal of inhibitors.

3rd stage. 5·82 l. of calcium phosphate gel (4·4% dry weight) were mixed with the dialysate, spun off and discarded, and a further 18 l. of gel were added to adsorb nearly the whole of the enzyme. The gel was centrifuged down and mixed with 700 g. $(NH_4)_2SO_4$ and 240 g. $NaHCO_3$, the final pH being 7·5. Elution was then effected by extracting four times with $(NH_4)_2SO_4$ solution (10 g./100 ml. water). Eluate: 18·7 l.; 1,084,000 units.

4th stage. 5·3 kg. $(NH_4)_2SO_4$ were added to the eluate, and after standing for 2 days at 0° the precipitate was spun off and discarded. A further precipitate was obtained by addition of 1 kg. $(NH_4)_2SO_4$ to the supernatant, and this was filtered off on a Buchner funnel, dissolved in water and dialyzed. Dialysate: 585 ml.; 515,000 units; 192 units/mg. N.

5th stage. 200 ml. of phosphate gel were added, spun off and discarded, involving a loss of 59,000 units. The supernatant was fractionated by adding solid $(NH_4)_2SO_4$ and the precipitates filtered off, dissolved and dialyzed free of salt:

Fraction		
1	172,000 units at 405 units/mg. N; 60%	Saturation with $(NH_4)_2SO_4$
2	152,000 units at 675 units/mg. N; 62%	
3	151,000 units at 340 units/mg. N; 64%	
4	43,000 units at 230 units/mg. N; 66%	
5	24,000 units at 112 units/mg. N; 70%	

6th stage. Fractions 1, 2 and 3, containing 455,000 units, were combined, dialyzed, adsorbed on 300 ml. of phosphate gel, and eluted as before. Eluate: 690 ml.; 362,000 units.

7th stage. The eluate was again fractionated with $(NH_4)_2SO_4$, the fourth fraction, obtained between 62 and 64% saturation, having the highest activity: 109,000 units; 790 units/mg. non-dialyzable N; total protein N, 0·14 g.

8th stage. This purified fraction after dialysis was brought to pH 5 with N/70-HCl, dialyzed for 4 hr., re-acidified to pH 5, and the precipitate thus formed spun off and discarded. The supernatant was neutralized to pH 6·5 and centrifuged clear. Supernatant: 82 ml.; 91,000 units; 835 units/mg. N.

This preparation represents a 4·6% recovery and a 133-fold purification from the yeast autolysate stage. It was stored under concentrated $(NH_4)_2SO_4$ solution and was quite stable at 0°. Although crystallization has not been effected, the electrophoresis of a 1·2% solution in 0·05 M-phosphate buffer pH 6 showed a single boundary over a period of 3 hr.; thereafter a trace of a slow-moving component could just be detected. At pH 6, the protein was negatively charged, and the mobility of the main ascending boundary was $3{\cdot}1 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$.

PROPERTIES

Activation by metallic cations. The enzyme is quite inactive except in the presence of the Mg ion, which cannot be replaced by the ions Ca, Mn, Zn or Be. Under the conditions of the standard test, the activation is maximal in 0·002 M-$MgCl_2$ and does not change if the Mg^{++} concentration is increased 20-fold. Fig. 1, illustrating these results, shows that 50 % activation is obtained with a Mg^{++} concentration of 0·0007 M.

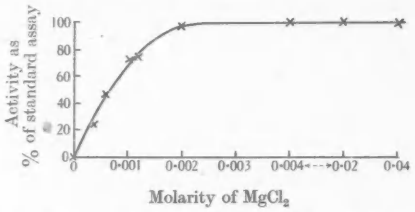


Fig. 1. Activation of yeast pyrophosphatase by Mg^{++} in M/35 veronal-acetate buffer pH 7·2 and M/2500-$Na_4P_2O_7$.

Specificity. The enzyme appears to catalyze specifically the reaction

$$HP_2O_7^{=} + H_2O \rightarrow 2HPO_4^{-} + H^+.$$

α-Glycerophosphate, hexosediphosphate, Na metaphosphate, Na triphosphate and ATP when tested under the conditions described for Na pyrophosphate were not attacked. Potassium diphenylpyrophosphate was tested manometrically (using sodium pyrophosphate as control) and flavinadenine dinucleotide by its ability to act as prosthetic group to *d*-amino-acid oxidase before and after treatment with pyrophosphatase. These substrates likewise remained intact. It thus appears that the pyrophosphate radical cannot in any way be substituted, either by a further esterification with phosphate (as in Na triphosphate and ATP) or by organic radicals (as in diphenylpyrophosphate and flavinadenine dinucleotide). It may be noted that various authors (Neuberg & Wagner, 1926; Kurata, 1931; Takahashi, 1932), using crude enzyme preparations from animal sources, have reported the breakdown of diphenylpyrophosphate; the inability of the purified yeast enzyme to do so was not unexpected in view of its inactivity towards ATP and Na triphosphate.

pH *activity curve.* The buffers employed were the Michaelis veronal-acetate mixture (M/35) and carnosine (M/20). pH values were measured in the glass electrode at 20° and corrected where necessary for the pH-temperature effect, since incubations were carried out at 38°. The reaction mixture (4·5 ml. of buffer, 0·2 ml. M/10-$MgCl_2$, 0·1 ml. enzyme and 0·2 ml. M/100 pyrophosphate) was incubated for 15 min., and the orthophosphate liberated at various pH values compared with that obtained in the standard veronal buffer at pH 7·2. In the pH range in which veronal and carnosine buffers overlap, the activity of the enzyme was similar in the two buffers, showing an optimum at pH 6·9–7·0 (Fig. 2), agreeing with that found by Bauer (1936) for the pyrophosphatase of bottom yeast, and also similar to that found by Lohmann (1928) for the pyrophosphatase of muscle extract. The curve indicates that the activity of the enzyme extends even beyond the explored pH range of 5·7–8·8.

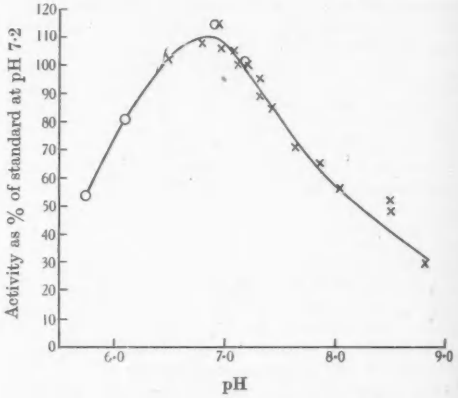


Fig. 2. pH-activity curve of yeast pyrophosphatase. Buffers: M/35 veronal-acetate (crosses) and M/20 carnosine (circles) containing M/250-$MgCl_2$ and M/2500-$Na_2P_4O_7$.

In animal tissues, several pyrophosphatases with differing pH optima have been reported (Takahashi, 1932; Minniti, 1939; Bamann & Gall, 1937). The last authors obtained from liver three such enzymes which were separated one from another, showing optima at pH 4, 5·7 and 8. Even in the crudest yeast extract, only the pyrophosphatase with optimum pH 7 has been reported (cf. Malkov & Kal, 1939).

Progress curve and effect of substrate concentration. The affinity of the enzyme for its substrate is too great to permit the direct measurement of initial hydrolysis rates at the very low pyrophosphate concentrations necessary to reduce the enzyme activity below the optimal. Using, for example, a pyrophosphate concentration of 0·0004 M, hydrolysis is linear with time until the reaction is 80 % complete, and falls to about 50 % of the maximum velocity when hydrolysis is about 93 % complete (Fig. 3). These results suggest a Michaelis constant of not more than 3×10^{-5}: such a high affinity is, we believe, unique for a hydrolyzing enzyme.

The activity of the enzyme shows a surprising sensitivity to increase of substrate concentration,

and begins to decrease when the pyrophosphate concentration exceeds 0·003 M (Fig. 4). The velocity of hydrolysis falls to 50 % of the maximal in 0·01 M-substrate, and to zero in 0·03 M. This effect, which

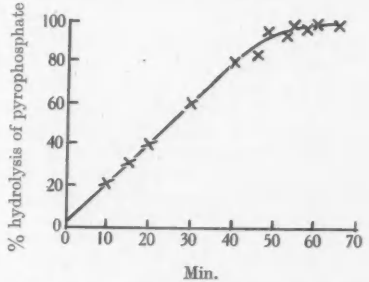


Fig. 3. Progress curve of $Na_4P_2O_7$ hydrolysis by pyrophosphatase. M/250-$MgCl_2$, M/35 veronal-acetate pH 7·2, initial $Na_4P_2O_7$ concentration M/2500.

is not abolished by a 10-fold increase in the Mg^{++} concentration, and is therefore not due to substrate-activator combination, is in fact a true inhibition, reversed simply by diluting. When, for example, the enzyme is incubated with M/250 substrate, no hydrolysis occurs, but after diluting 10–20 times (maintaining the Mg^{++} concentration at its previous value) enzyme action proceeds at the maximal rate. This complete inhibition of an enzyme by its sub-

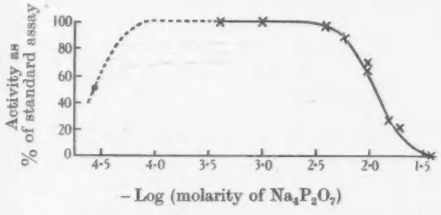


Fig. 4. Effect of substrate concentration on pyrophosphatase activity. M/250-$MgCl_2$, M/35 veronal-acetate buffer pH 7·2. Dotted portion inferred from progress curve, Fig. 3.

strate in comparatively low concentration appears to be unique. In other cases, e.g. liver esterase (Bamann, 1929) and urease (Howell & Sumner, 1934), the activity decreases at fairly high substrate concentrations, but the inhibition is never complete. (The activity-substrate concentration curves for xanthine oxidase, reported by Dixon & Thurlow (1924), are similar to that for pyrophosphatase; the analogy is, however, only superficial, since the effect of substrate on the oxidase is due to competition of xanthine or of hypoxanthine with the methylene blue used as hydrogen acceptor.)

It must be noted here that this substrate effect necessitated the use of low concentrations (M/250–M/1000) of potential substrates in the foregoing specificity experiments.

The effect of other cations on Mg-*activated pyrophosphatase.* The activity of Mg-activated pyrophosphatase was found by Bauer (1937) to decrease in presence of Ca ions, an effect partially overcome by raising the Mg^{++} concentration. This type of competitive inhibition appears from the work of Greville & Lehmann (1943, 1944) to be applicable to other enzyme systems requiring a divalent ion activator. Thus the Ca-activated adenosine triphosphatase activity of myosin and the Ca-activated process of plasma clotting are both inhibited by Mg ions; conversely, the Mg-activated adenosine triphosphatase activity of electrical tissue is inhibited by Ca ions.

The effect of a number of ions on the purified yeast pyrophosphatase in presence of Mg^{++} has been examined. The experimental conditions were similar to those of the standard test; where necessary, reagents added were first neutralized to the pH of the buffer. Table 1 shows that, at low pyrophosphate

Table 1. *Inhibition of* Mg-*activated pyrophosphatase by cations*

(Standard test conditions, 0·004 M-$MgCl_2$)

$Na_4P_2O_7$ (M)	Ionic ratio [cation]/[Mg]	Percentage inhibition				
		Ca	Zn	Mn	Al	Be
0·0004	1·0	100	100	100	—	100
0·0004	0·1	68	84	86	—	78
0·002	0·5	90	84	77	70	76
0·004	0·1	69	40	20	20	10

concentrations, all ions tested act as effective inhibitors; by increasing the substrate concentration, the effect of Mn^{++}, Al^{+++}, Be^{++} is largely abolished, with that of Ca^{++} remaining unchanged and Zn^{++} occupying an intermediate position. These data are explained by postulating two effects: the one, manifest at low substrate concentrations and due to combination of ion and substrate, such that activity is reduced by substrate depletion; the other, a true inhibition, due to a competition with Mg^{++} for the active centre of the enzyme, as demonstrated by Ca^{++} and to some extent by Zn^{++}. These latter metals are closely related to Mg in the periodic table. The unequivocal resolution of the two effects would probably be achieved at still higher substrate concentrations, but, as already shown, the enzyme is then inhibited by its substrate.

When the Ca effect was examined more systematically, using 0·0004 M-pyrophosphate and (*a*) constant $[Mg^{++}]$ with varying $[Ca^{++}]$, or (*b*) variable $[Mg^{++}]$ with constant $[Ca^{++}]$, the activities were found to depend entirely on the $[Ca^{++}]/[Mg^{++}]$

ratio. All points from all series of experiments thus fall on the one curve of Fig. 5, showing convincingly the competition which exists between Ca^{++} and Mg^{++} for some active group. From the curve, the remarkable effect of Ca^{++} is clearly shown; when, for example, the Ca^{++} concentration is only half that of Mg^{++}, the enzyme is completely inactive, and even a Ca^{++}/Mg^{++} ratio of 1:10 produces a 65% inhibition.

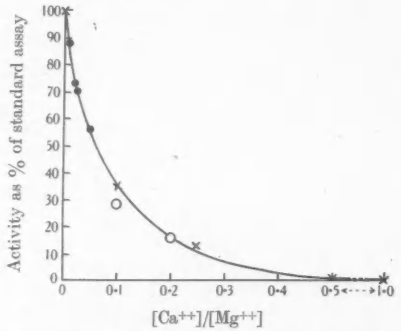


Fig. 5. Pyrophosphatase activity as a function of $[Ca^{++}]/[Mg^{++}]$ ratio. M/2500-$Na_4P_2O_7$, M/35 veronal-acetate buffer pH 7·2. Cation concentrations: 0·004M-Mg^{++} and varying Ca^{++} (crosses); 0·004M-Ca^{++} and varying Mg^{++} (circles); 0·0004M-Ca^{++} and varying Mg^{++} (dots). In all cases sufficient Mg^{++} was present to activate the enzyme completely in absence of Ca^{++}.

Inhibitors. The inhibitor after dissolving in water was added to the enzyme diluted under the conditions of the standard activity assay. After 15 min. at 38°, the pyrophosphate was added to a concentration of 0·0004M. The most powerful inhibitors, NaF, Cu^{++} (as sulphate), and $CH_2ICOONa$ gave 50% inhibition at molarities of 2×10^{-5}, 4×10^{-5}, and 5×10^{-3} respectively. Metaphosphate (0·01M) and cyanide (0·002M) were without effect.

The inhibition produced by alloxan (50% with 0·01M-reagent) is readily reversed by addition of 0·04M-cysteine, but not by KCN—results which suggest that SH groups are essential for the enzyme activity (cf. Hopkins, Morgan & Lutwak-Mann, 1938). In this connexion it was found that crude pyrophosphatase preparations were more active by some 20% in 0·02M-cysteine. The purified enzyme, freshly diluted, does not respond to activation in this way, but when the activity is allowed to fall by about 40% after the enzyme is stored in high dilution at pH 7 and 0° for 2 weeks, a partial reactivation occurs both in presence of cysteine and of KCN. The inhibition of the enzyme by iodoacetate gives further evidence of its SH character.

SUMMARY

1. The pyrophosphatase of baker's yeast has been purified. The preparation, having at 38° a Q_P value of 10^5, showed only a trace of impurity in the Tiselius electrophoretic apparatus.

2. Inhibition and reversal experiments suggest that pyrophosphatase is an SH enzyme, specifically activated by the Mg ion.

3. Specificity studies indicate that only inorganic pyrophosphate is attacked, the optimal pH being 6·9–7·0.

4. The enzyme has a very high affinity for its substrate (Michaelis constant *c.* 3×10^{-5}), but is completely inactive in substrate concentrations greater than 0·03M, an effect reversible by dilution.

5. The activation by Mg^{++} is antagonized by Ca^{++} to an extent depending only on the $[Ca^{++}]/[Mg^{++}]$ ratio.

The authors are indebted to Dr M. Dixon for his continued advice and criticism, and to the Director General of Scientific Research and Development, Ministry of Supply, for permission to publish this paper.

REFERENCES

Bailey, K. (1942). *Biochem. J.* **36**, 121.
Bamann, E. (1929). *Ber. dtsch. chem. Ges.* **62**, 1538.
—— & Gall, H. (1937). *Biochem. Z.* **293**, 1.
Bauer, E. (1936). *Hoppe-Seyl. Z.* **239**, 195.
—— (1937). *Hoppe-Seyl. Z.* **248**, 213.
Dixon, M. & Thurlow, S. (1924). *Biochem. J.* **18**, 976.
Fleury, P. & Courtois, J. (1937). *Enzymologia*, **1**, 377.
Greville, G. D. & Lehmann, H. (1943). *Nature, Lond.*, **152**, 81.
—— —— (1944). *J. Physiol.* **103**, 175.
Hopkins, F. G., Morgan, E. J. & Lutwak-Mann, C. (1938). *Biochem. J.* **32**, 1829.
Howell, S. F. & Sumner, J. B. (1934). *J. biol. Chem.* **104**, 619.
Huber, H. (1937). *Z. angew. Chem.* **50**, 323.
Kay, H. D. (1928). *Biochem. J.* **22**, 1446.
Kurata, K. (1931). *J. Biochem., Tokyo*, **14**, 25.
Lohmann, K. (1928). *Biochem. Z.* **203**, 172.
Malkov, A. M. & Kal, V. V. (1939). *Ukr. biochem. J.* **13**, 633.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Minniti, G. (1939). *Biochim. Terap. sper.* **26**, 417.
Neuberg, C. & Wagner, J. (1926). *Biochem. Z.* **171**, 485.
Roche, J. (1931). *Biochem. J.* **25**, 1724.
Takahashi, H. (1932). *J. Biochem., Tokyo*, **16**, 447.

The Detection and Estimation of Uric Acid by 2:6-Dichloroquinone-chloroimide

By W. R. FEARON, *Department of Biochemistry, Trinity College, Dublin*

(*Received* 4 *August* 1944)

When a very dilute (0·001–0·05 %) solution of uric acid is made slightly alkaline by addition of a pH 9–10 buffer, and treated with two to three drops of 0·4 % 2:6-dichloroquinone-chloroimide in ethanol, the mixture rapidly develops a bright yellow colour, which becomes red on addition of a few drops of 1 % silver nitrate. If the uric acid be in excess, the secondary colour change may be obscured by the formation of the dark brown silver reduction complex described by Schiff as a test for uric acid.

Although many biological compounds give colours with the chloroimide reagent in alkaline solution, cysteine and reduced glutathione were the only ones found to yield yellow pigments, and these differ from the uric acid pigment in that they are not turned red by Ag^+. Less oxidized purines, such as hypoxanthine and xanthine, give no colour with the reagent. With 20 ml. of solution, the dilution limit of the test for uric acid is about 1 in 10^6, and is of the same order of delicacy as the phosphotungstate reduction test developed by Folin (1922, 1934).

EXPERIMENTAL

Scope of the chloroimide reagent. The reaction with uric acid was observed while urine was being tested for free phenols by the method of Gibbs (1927 *a*, *b*), who has shown that the chloroimide reagent is a general test for phenols unsubstituted in the *para*-position, with which it forms blue or violet indophenol pigments. The reagent also has been used by Raybin (1938) for the detection of thiamine, and by Scudi (1941) for the estimation of pyridoxin.

Scudi reports that creatine, creatinine, and phenylhydrazine give a yellow, uric acid and carbonbisulphide a yellow to pink, and furfuraldehyde a green colour with the buffered reagent. This has not been confirmed for a specimen of pure creatine, and the validity of the colour tests has been found to depend on several factors: the pH of the mixture, the form in which the reagent is added, the presence or absence of metallic catalysts, and the time allowed for reaction. For most purposes pH 9–10 is the best buffer range. At values below pH 8 colour development is slow, and the shade of the uric acid pigment is orange, although the test can be demonstrated by buffering the solution by addition of solid sodium acetate. In solutions above pH 10, the reagent tends to undergo spontaneous decomposition, with production of a red-brown mixture of pigments. Gibbs has found that this decomposition is accelerated by exposure to light, as well as by increasing alkalinity. During the present work, it has been

Table 1. *Colour reactions of various substances with 2:6-dichloroquinone-chloroimide at* pH 9·2

Amino compounds:	
NH_3	No change
Methylamine	Slow purple, stable
Glycine	Slow violet, stable
Alanine	No change
Cystine	No change
Tyrosine	No change
Tryptophan	No change
Proline	Slow purple, turns brown
Arginine	No change
Histidine	Slow purple, turns brown
Ornithine	Slow purple, turns brown
Sulphur compounds:	
H_2S	Transient red
KSCN	No change
Thiourea	Slow violet, catalyzed by Cu^{++}
Formamidine disulphide	Rapid violet
Cysteine	Rapid yellow
Glutathione, reduced	Rapid yellow
Purines:	
Hypoxanthine	No change
Xanthine	No change
Guanine	No change
Uric acid	Rapid yellow, turned red by Ag^+
Caffeine	No change
Water-soluble vitamins:	
Thiamine	Slow orange-red, soluble in chloroform
Riboflavin	No change
Pyridoxin	Red-violet
Inositol	No change
Ascorbic acid	No change
Urinary solutes:	
Urea	No change
Creatine	No change
Creatinine	Slow red, turns brown
Hippuric acid	No change
Allantoin	No change
Miscellaneous:	
Adrenaline	Transient red
Indole	Slow violet
Phenols, substituted in *para*-position	No change
Phenols, unsubstituted	Blue or violet

found possible to repress the decomposition of the reagent by incorporating 10 % of NaCl in the buffer mixture.

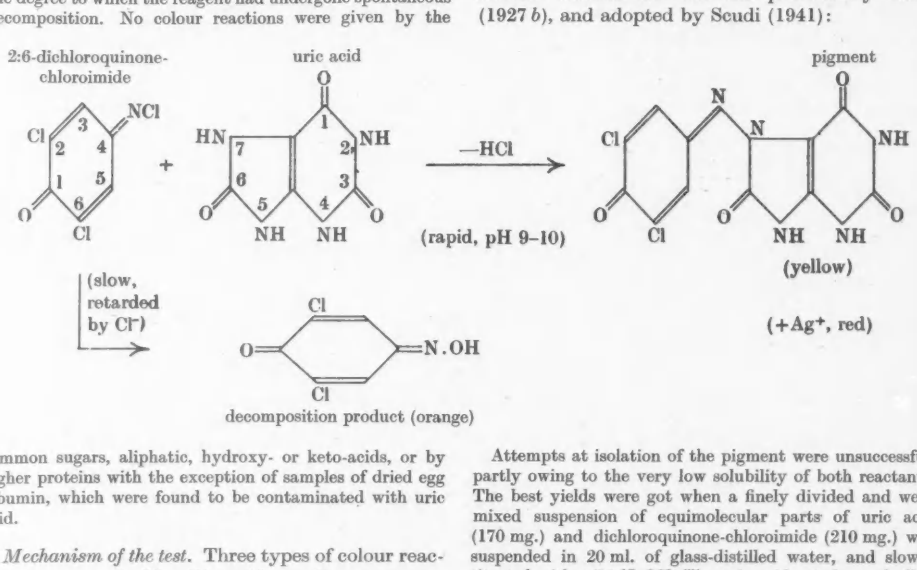
Either the 2:6-dibromo- or the 2:6-dichloroquinone can be used as the reagent, the latter being chosen on account of its greater stability in the buffer-chloride mixture. Owing to the very low solubility of both reagents in water they were applied as 0·4 % solutions in aldehyde-free 98 % ethanol. This is a higher concentration than that used by Scudi, as it was observed that excess of ethanol added with the reagent promoted decomposition during the test.

To ascertain the scope of the reaction, the following tests were carried out by mixing 2 ml. of a pH 9·2 borate buffer with 2 ml. of a 0·01–0·1 % solution of the compound examined. Two drops of the 0·4 % reagent were added, and 5 min. allowed for colour development. The results are shown in Table 1. The reactions with methylamine, glycine, thiourea and indole were accelerated by shaking and aeration, though this promoted the decomposition of the reagent in the tests with proline, histidine and ornithine. On acidification with glacial acetic acid the phenolic colours were changed to bright red, the colours given by methylamine and glycine were changed to a stable violet, and the other pigments were more or less bleached, depending on the degree to which the reagent had undergone spontaneous decomposition. No colour reactions were given by the common sugars, aliphatic, hydroxy- or keto-acids, or by higher proteins with the exception of samples of dried egg albumin, which were found to be contaminated with uric acid.

Mechanism of the test. Three types of colour reaction are recognizable: (1) rapid coupling, as occurs with the phenols, uric acid and some thiols; (2) slow interaction, probably accompanied by oxidation, as occurs with amino and imino compounds, and yields red or purple pigments closely alike in shade; and (3) spontaneous decomposition of the chloroimide with formation of orange-brown pigments that may mask the original colour reaction. When this is suppressed by the use of a buffer-chloride mixture, the original colours are much more stable and characteristic; that given by uric acid persists for at least an hour unchanged, as shown by absorptiometer measurements.

Comparison of the formulae suggests that the test for uric acid requires the presence of a labile H in position 7, adjacent to the OH at 8, and the double bond at 4–5. This, as Biltz (1936) has shown, constitutes a reacting system altogether unlike that in the less oxidized purines. Under appropriate conditions condensation appears to involve only one molecule of each of the reactants, and HCl is liberated in the process. It is independent of oxidation, although, once the pigment has formed, it can be reversibly bleached by reducing agents.

If the yellow pigment is formed by condensation between the chloroimide grouping in the quinone and the NH— at position 7 in the purine ring, the subsequent change to red on addition of Ag^+ can be explained as an example of the silver-binding NH group effect resembling that shown by the substituted rhodanines (Feigl, 1940). This condensation can be formulated in accordance with the reaction scheme worked out for the phenols by Gibbs (1927 *b*), and adopted by Scudi (1941):



Attempts at isolation of the pigment were unsuccessful, partly owing to the very low solubility of both reactants. The best yields were got when a finely divided and well-mixed suspension of equimolecular parts of uric acid (170 mg.) and dichloroquinone-chloroimide (210 mg.) was suspended in 20 ml. of glass-distilled water, and slowly titrated with N/10-NaOH. The uric acid acted as a buffer, and the mixture was kept in the region of pH 8–8·5 by continual stirring and slow addition of the alkali. At such pH the colour of the pigment is a deep orange, changing to yellow when a drop of the filtrate is added to 5 ml. of pH 10 buffer. Although it was possible to obtain very concentrated solutions of the pigment by this method, all underwent further changes during attempts at crystallization.

ESTIMATION OF URIC ACID IN URINE

Owing to the excess of chloride in the buffer mixture employed, the red pigment cannot be satisfactorily developed by addition of Ag^+, but the primary yellow pigment is sufficiently intense and stable to be used for the colorimetric estimation of uric acid in concentrations down to 1 in 100,000.

Reagents

pH 10 *buffer-chloride mixture.* Add 43·9 ml. of N/5-NaOH to 50 ml. of 2M-boric acid in 0·2M-KCl, and make up to 200 ml. with 20% NaCl, using copper-free distilled water and A.R. reagents.

Chloroimide reagent. 0·4% 2:6-dichloroquinone-chloroimide (British Drug Houses Ltd.) in aldehyde-free absolute ethanol. Kept in brown glass bottles, and stored in the dark, the reagent is stable for at least 3 months. Exposed to daylight, deterioration begins within 12 hr., and the solution darkens on being mixed with the buffer. Similar results were obtained with a purified sample of the reagent, synthesized by Prof. T. J. Nolan.

Uric acid standard. The stock solution, containing 1 g./l., is prepared according to Folin's method (1922), which is in general use. The stock solution, when compared with freshly prepared standards, was found to have retained its original value after storage for a year in the dark. Uric acid standards prepared by other methods deteriorated within a month. The colorimetric standard, containing 1 mg. uric acid/100 ml., is prepared freshly as required, by diluting 1 ml. of stock to 100 ml., with glass-distilled water. It does not keep for more than a day or two.

Method

For the concentration range of 10–80 mg. uric acid/100 ml., which includes all values normally found in human urine, the urine is diluted 1 in 50 with distilled water. Five ml. of the diluted urine are transferred to a 10 ml. measuring flask, and mixed with 1 ml. of pH 10 buffer-chloride solution and 0·05 ml. of the 0·4% chloroimide reagent. Within a minute the mixture is diluted to 10 ml. with distilled water, and, within 5–10 min., compared with a standard prepared in the same way, 5 ml. of a solution containing 1 mg. uric acid/100 ml. being used. Readings are taken in the ordinary type of colorimeter, the standard being set at 20 mm. Alternatively, readings can be taken by means of the Hilger Spekker absorptiometer, with the violet glass filter no. 7.

Calculation. $X = 50 \times S/U$,

where X is uric acid in mg./100 ml. in the original undiluted urine, S is the reading of the standard ($S = 20$), U is the reading of the unknown solution.

Urines very rich in uric acid may require to be diluted 1 in 100.

RESULTS

Recovery. Recovery values, obtained by adding various amounts of the stock solution to urine, showed that the method gives consistent results within the concentration range of uric acid found in ordinary urines (Table 2).

Table 2. *Recovery values for uric acid in urine*

Uric acid added (mg./100 ml.)	Uric acid recovered (mg./100 ml.)	Recovery (%)
10	9·99	99·9
20	19·92	99·6
30	29·89	99·6
40	39·78	99·4
50	49·90	99·8

Interfering substances. Urinary solutes capable of interfering with the reaction are: (1) amino compounds, including creatinine and free amino-acids; (2) thiols, including cysteine and reduced glutathione; (3) free phenols unsubstituted in the *para*-position. The concentrations of all these, with the exception of creatinine, and, occasionally, histidine and phenols, are so low that their effects are completely suppressed by diluting the urine 1 in 50; while at this dilution, creatinine reacts too slowly to interfere, if the estimation is completed within 10 min. Although early morning samples of pregnancy urine may contain 30–100 mg. of histidine/100 ml. (O'Brien & Quelch, 1943), specimens of pregnancy urine rich in histidine gave uric acid colours that could be matched without difficulty, and addition of 100 mg. of histidine to 100 ml. of normal urine did not obviously affect the uric acid value. This, again, is probably due to the relative slowness of the amino-acid reaction. Interference due to phenols is shown by the development of a green colour during the test. The phenolic reaction is very delicate, and can only be eliminated by extraction of the acidified urine with ether before carrying out the estimation. This is rarely necessary, as *p*-cresol, the commonest urinary phenol, does not give a colour with the reagent, and the other phenols are almost entirely in esterified non-reacting form in fresh urine. It appears doubtful if free phenols occur in significant amounts in ordinary urine, unless hydrolysis has taken place on keeping. The commoner pathological constituents: proteins, reducing sugars, acetone and acetic acid, do not interfere with the estimation. Bile pigments, as might be expected, obscure the reaction.

Diurnal variation. To see if colour development was modified in any way by variations in the composition of urine during the day, an analysis was made of each 2-hourly specimen over a period of 24 hr. No difficulty was found in matching the colours in the ordinary colorimeter, although, in the early morning specimens, where the uric acid output was very low, development of the full colour was slightly delayed (Table 3).

State of uric acid in urine. Since the chloroimide method is very rapid, and involves no drastic treatment of the urine, it should reveal if the uric acid is present as a simple solute, or is, in part at least, esterified or otherwise bound. This was investigated

Table 3. *Two-hourly output of uric acid in urine*

Time	Vol. (ml.)	Sp. gr.	pH	Uric acid (mg./100 ml.)	Uric acid (mg.)
12 noon	350	1005	6·4	14·8	51·8
2 p.m.	74	1018	7·4	41·7	31·3
4 p.m.	263	1006	5·8	20·0	52·6
6 p.m.	153	1010	6·0	32·7	49·1
8 p.m.	130	1012	5·8	34·5	45·0
10 p.m.	158	1012	6·6	33·0	39·5
12 p.m.	130	1011	6·6	37·9	50·5
2 a.m.	86	1015	5·4	33·0	26·4
4 a.m.	54	1018	5·2	35·4	17·8
6 a.m.	43	1020	5·2	45·4	19·5
8 a.m.	40	1020	5·4	55·5	22·2
10 a.m.	44	1018	5·4	53·5	23·1
		Total, 10 a.m. to 10 a.m.			428·8

by comparing the uric acid values of fresh samples with samples kept for 24 hr. in presence of dilute acid and alkali. As the results were almost identical, it appears that uric acid in urine occurs as a simple solute (Table 4).

Table 4. *Uric acid values of urine after treatment with dilute acid or alkali at* 18°

Urine	Uric acid (mg./100 ml.)	
Fresh, pH 5·8	30·5	30·3
After 24 hr., undiluted	30·1	30·3
After 24 hr., diluted with an equal vol. of water	30·3	30·6
After 24 hr., diluted with an equal vol. of N/10-HCl	30·12	29·94
After 24 hr., diluted with an equal vol. of N/10-NaOH	30·5	30·3

DISCUSSION

Validity of the method. In general, the values obtained for urinary uric acid by the chloroimide method are of the same order, but slightly lower than values obtained by the phosphotungstate reduction methods. Variations, however, often occurred in the results obtained by different clinical workers using different preparations of the phosphotungstate and cyanide reagents, and it was concluded that unless the entire group of methods was checked by the actual isolation and direct estimation of the uric acid present in the samples, conclusions could not be safely established. This procedure was adopted by Myers & Wardell (1928), but present circumstances did not allow of it being attempted with prospect of the same degree of accuracy as that postulated by the American workers. The exhaustive survey by Brøchner-Mortensen (1937) shows that any comparison of values for urinary uric acid depending on colorimetry can be misleading, as the same set of errors may reappear in different methods, notably those involving tungstate reduction, which, according to Folin (1934), 'represents probably the most complex reaction we have in the whole field of practical colorimetry'.

SUMMARY

1. In buffer solutions at pH 9–10, uric acid reacts with 2:6-dichloroquinone-chloroimide, forming a yellow pigment that turns red on addition of Ag^+.

2. The test can be used for the estimation of uric acid in concentrations down to 1 in 100,000, and has been adapted for urine analysis.

I wish to thank Prof. T. J. Nolan for a pure preparation of 2:6-dichloroquinone-chloroimide. I am indebted to Dr E. Kawerau for having carried out the experiment on the 2-hourly excretion of uric acid, and to Mr J. Harwood for having done most of the estimations.

REFERENCES

Biltz, H. (1936). *Die neure Harnsäurechemie.* Leipzig: Barth.

Brøchner-Mortensen, K. (1937). *Uric Acid in Blood and Urine.* Copenhagen: Levin and Munksgaard.

Feigl, F. (1940). *Specific and Special Reactions.* New York: Elsevier.

Folin, O. (1922). *Laboratory Manual of Biological Chemistry.* New York and London: Appleton.

Folin, O. (1934). *J. biol. Chem.* **106**, 311.

Gibbs, H. D. (1927*a*). *J. phys. Chem.* **31**, 1053.

—— (1927*b*). *J. biol. Chem.* **72**, 649.

Myers, V. C. & Wardell, E. L. (1928). *J. biol. Chem.* **77**, 697.

O'Brien, J. R. & Quelch, P. E. (1943). *Biochem. J.* **37**, *Proc.* xvii.

Raybin, H. W. (1938). *Science*, **88**, 35.

Scudi, J. V. (1941). *J. biol. Chem.* **139**, 707.

Some Hemicellulose Extraction Methods

By I. A. PREECE, *Chemistry Department, Heriot-Watt College, Edinburgh*

(*Received* 4 *August* 1944)

Hemicellulose preparations made by methods involving extraction of woody tissues with aqueous sodium hydroxide are mixtures, containing materials resistant to chlorination and others which are rendered soluble in sodium sulphite solution by this treatment. The two types have tentatively been designated cellulosan and encrusting hemicelluloses (Norman, 1937; Preece, 1941), and their proportions

in a given preparation depend on the actual conditions of extraction. It is clear that, to obtain a useful knowledge of the nature of each, separate extraction is necessary, and since it has been shown that delignification facilitates hemicellulose extraction (see, e.g., Norman & Shrikhande, 1935; Norman, 1937) the suggestion arises that holocellulose would provide the ideal starting material. Thus, Mitchell & Ritter (1940) have successively extracted holocellulose with water, dilute sodium carbonate solution, and sodium hydroxide solution; however, this does not take into account the dual origin of hemicellulose fractions, whilst the use of hot alkaline solutions for extraction is inadmissible owing to the certainty of hemicellulose degradation thereby (Preece, 1940).

A general consideration of the position suggests points which must receive attention in attempting a separation. Preliminary extraction with boiling 0·5% (w/v) ammonium oxalate solution and with water is desirable to remove pectin and water-soluble materials; suitable means of delignification should then be sought, but the procedure must not itself involve any significant loss of furfuraldehyde-yielding material nor must it diminish the yield, or in any way modify the crude cellulose as determined on the wood residue or holocellulose and compared with that from the original wood. In the most favourable case complete extraction of encrusting hemicellulose might then be possible with water, though other solvents (e.g. 1% (w/v) sodium sulphite solution) could be used if it were shown that they have no degradative effect. Again such extraction must not influence the yield or composition of the crude cellulose. Careful control is necessary at all stages, so that the origin and fate of all furfuraldehyde-yielding material is known. The possibility of separately extracting encrusting hemicelluloses following simple delignifying methods, not necessarily leading to holocellulose production, is examined below.

METHODS

Since large-scale working is ultimately to be aimed at, it seems preferable to use a modification of the Norman & Jenkins (1933) hypochlorite technique in those cases where chlorination is to be applied. Comparative determinations require to be carried out on individual samples and not successively on the same sample, since oven-drying apparently increases the solubility of certain of the cellulosic materials present (Norman, 1936). Willow sawdust was used, as this material had proved to be rich in encrusting hemicelluloses (Preece, 1941) and to yield preparations the compositions of which are very susceptible to conditions of working; a new sample of the sawdust being used, results are not directly comparable with those quoted earlier. All determinations were carried out on 2 g. portions of the sawdust, which had been previously extracted in bulk with boiling 0·5% (w/v) ammonium oxalate solution and with water and air-dried at room temperature; the necessary adjustments for the moisture contents of the original wood and of the air-dried residue are made in the calculations.

Chlorination. The extracted wood is washed into a beaker with 100 ml. of 0·2N-H_2SO_4, 5 ml. of sodium hypochlorite solution (approx. 12% (w/v) of available chlorine) are added, and the mixture is stirred for 7 min. with the beaker standing in a vessel of water to maintain the temperature within the range 14–18°. The volume of acid is just sufficient to liberate substantially the whole of the chlorine. The wood is then filtered at the pump and well washed with water. If the immediately following treatment is to be with an ethanolic reagent, the residue is further washed with 80% ethanol.

Alkaline treatments and pretreatments. The wood, with or without preceding chlorination, is washed into the beaker with 40 ml. of the ethanolic alkaline reagent, and treated with stirring at controlled temperature for 7 min. as before, except in the cases of some experiments summarized in Tables 3, 4 and 5, where the alkaline treatment was for longer times. After filtration and washing with 80% ethanol, the residue is available for any desired further treatment. After the final alkaline treatment of a series, the ethanolic washing is in some cases followed by washings with ethanolic H_2SO_4 (5 g. of H_2SO_4/100 ml. of 80% ethanol) and aqueous 0·2N-H_2SO_4 (in that order when both are used).

Aqueous extraction of residue. The residue is twice extracted under reflux for ½ hr. periods with 100 ml. portions of water, extracts and washings being combined and concentrated for further examination.

Sulphite extraction. This normally follows aqueous extraction and is carried out similarly, using 100 ml. portions of boiling 1% (w/v) sodium sulphite ($Na_2SO_3.7H_2O$) solution.

Crude cellulose determination. The procedure for chlorinations is precisely that described above, but the subsequent sulphite extractions are for 20 min. each, with 80 ml. portions of 3% (w/v) sodium sulphite solution in a boiling water-bath.

Lignin determination. Lignin was not determined separately as such, owing to the uncertainty attaching to results obtainable by the usually available methods. However, a measure of the degree of delignification is available for many of the treatments by subtracting the figure for the yield of wood residue from 94·92, the value for wood residue after extractions with ammonium oxalate and water (Table 1). The figure for delignification so obtained will slightly over-estimate the true value, owing to the loss of a small proportion of furfuraldehyde-yielding material (see below); it will, however, serve as a useful guide to the progress of lignin removal.

RESULTS

As a measure of the efficiency of extraction, furfuraldehyde yields were determined on wood residues, aqueous and sulphite extracts and crude cellulose. Further, the yields of wood residue and crude cellulose (both ash-free) are also quoted. For ease of comparison, all results (unless otherwise stated) are calculated to the original, dry, unextracted wood.

Table 1. *Influence of progressive alternation of chlorination and alkaline extraction of willow sawdust on the yield and composition of wood residue and crude cellulose and the solubility of furfuraldehyde-yielding components*

(Final washing includes use of ethanolic and aqueous H_2SO_4)

No. each of chlorinations and alkaline treatments...	No treatment 0	Treatment with 40 ml. of ethanolic NH_3* 2	4	6	8	10	Treatment with 40 ml. of ethanolic NaOH† 2	4	6
Wood residue (%)	94·92	93·38	87·85	83·45	77·04	74·97	70·84	66·84	66·27
Crude cellulose (%)	57·79	56·32	55·67	54·75	55·03	54·77	56·71	54·01	52·07
Furfuraldehyde from:									
(*a*) Wood residue (%)	14·42	14·30	14·18	13·95	13·50	13·41	13·62	12·95	13·08
(*b*) Aqueous extract (%)	0·00	0·70	1·26	1·76	2·33	2·39	3·11	3·95	5·16
(*c*) Sulphite extract (%)	0·21	2·93	3·46	3·37	2·93	2·92	0·92	1·04	0·96
(*d*) Crude cellulose (%)	9·18	8·79	8·35	7·85	7·99	7·98	8·63	7·15	6·26
(*b*+*c*)	0·21	3·63	4·72	5·13	5·26	5·31	4·03	4·99	6·12
(*b*+*c*+*d*)	9·39	12·42	13·07	12·98	13·25	13·29	12·66	12·14	12·38

(Results as percentage of original dry wood.)
* 10 ml. of ammonium hydroxide (sp. gr. 0·88) and 90 ml. of 80% ethanol.
† 4 g. of NaOH/100 ml. of 80% ethanol.

Table 2. *Changes in yield and composition of crude cellulose, and in the solubility of furfuraldehyde-yielding components of willow sawdust, resulting from various alkaline pretreatments*

(Six chlorinations and six alkaline pretreatments, without final acid washings)

Alkali used ...	NH_3* In 80% ethanol	NH_3* In 95% ethanol	NaOH 4% In 80% ethanol	NaOH 1% In 80% ethanol	Ethanolamine 3% In 95% ethanol	Ethanolamine 5% In 95% ethanol
Crude cellulose (%)	55·76	55·74	53·24	57·21	56·30	56·71
Furfuraldehyde from:						
(*b*) Aqueous extract (%)	2·05	1·95	5·12	3·11	1·40	1·42
(*c*) Sulphite extract (%)	2·91	3·03	0·17	0·19	2·70	2·69
(*d*) Crude cellulose (%)	8·11	7·89	7·14	9·16	8·17	8·35
(*b*+*c*)	4·96	4·98	5·29	3·30	4·10	4·11
(*b*+*c*+*d*)	13·07	12·87	12·43	12·46	12·27	12·46

(Results as percentage of original dry wood.)
* 10 ml. of ammonium hydroxide (sp. gr. 0·88) and 90 ml. of 80 or 95% ethanol.

Table 1 shows the progressive delignification and the increasing water-solubility of hemicelluloses which result from increasing numbers of 7 min. treatments with ethanolic NH_3 or ethanolic NaOH as delignifying agents following in alternation with an equal number of chlorinations. With NaOH, delignification is substantially complete after six pairs of treatments, whilst further treatment with NH_3 beyond this number does not increase the combined yields of furfuraldehyde from aqueous and sulphite extracts. Since, however, the partition of furfuraldehyde between the two extracts may be influenced by the final acid washings, the effect of these is shown by Table 2. In obtaining these results NH_3 and NaOH have each been used in two combinations with ethanol, and treatments have also been included using ethanolamine in two concentrations.

Ethanolic NaOH itself plays a big part in increasing the water-solubility of hemicelluloses, even though chlorination is not applied, as shown for single treatments in Tables 3 and 4. The further influences of variations in time of treatment, of different modes of washing prior to aqueous and sulphite extraction, and of changes in NaOH concentration, are also demonstrated.

Further light on the rendering soluble of hemicelluloses is thrown by the series of treatments and pretreatments summarized in Table 5, in all of which the alkaline reagent used was ethanolic NaOH (4 g./100 ml.).

(i) Sawdust was treated for four periods, each of 1 hr., with this reagent, each treatment being followed by washing with ethanol and ethanolic H_2SO_4 only, and by two extractions with boiling water. Four aqueous extraction fractions were thus obtained on each of which furfuraldehyde was determined.

(ii) A single 1 hr. treatment with ethanolic NaOH with washing and water extraction as in (i) was followed by chlorination (7 min.) and treatment with ethanolic (95%) ethanolamine (5 ml./100 ml.). Washing with ethanol and ethanolic H_2SO_4 was followed by extraction with boiling

Table 3. *Influence of a single pretreatment with ethanolic* NaOH* *(without chlorination) on the solubility of furfuraldehyde-yielding components of willow sawdust*

Washing prior to extraction ...	Ethanol, ethanolic H_2SO_4, aqueous H_2SO_4				Ethanol, ethanolic H_2SO_4				Ethanol only
Treatment time (hr.)	½	1	2	24	½	1	2	24	24
Wood residue (%)	—	—	—	—	—	91·05	—	—	—
Crude cellulose (%)	—	—	—	—	59·27	58·68	57·91	57·44	—
Furfuraldehyde from:									
(*a*) Wood residue (%)	—	—	—	—	—	14·28	—	—	—
(*b*) Aqueous extract (%)	1·38	1·90	1·94	2·54	1·98	2·37	2·63	2·87	3·19
(*c*) Sulphite extract (%)	0·48	0·40	0·71	0·74	0·19	0·18	0·18	0·20	0·00
(*d*) Crude cellulose (%)	—	—	—	—	10·18	10·04	9·92	9·80	—
(*b* + *c*)	1·86	2·30	2·65	3·28	2·17	2·55	2·81	3·07	3·19
(*b* + *c* + *d*)	—	—	—	—	12·35	12·59	12·73	12·87	—

(Results as percentage of original dry wood.)

* 4 g. of NaOH/100 ml. of 80% ethanol.

Table 4. *Influence of a single pretreatment with ethanolic* NaOH* *(without chlorination) on the solubility of furfuraldehyde-yielding components of willow sawdust*

Washing prior to extraction ...	Ethanol, ethanolic H_2SO_4, aqueous H_2SO_4			
Treatment time (hr.) ...	1	2	4	24
Crude cellulose (%)	59·08	58·12	57·65	57·91
Furfuraldehyde from:				
(*b*) Aqueous extract (%)	1·20	1·40	1·57	1·40
(*c*) Sulphite extract (%)	0·65	0·88	0·79	0·76
(*d*) Crude cellulose (%)	10·24	9·70	9·58	9·91
(*b* + *c*)	1·85	2·28	2·36	2·16
(*b* + *c* + *d*)	12·09	11·98	11·94	12·07

(Results as percentage of original dry wood.)

* 1 g. of NaOH/100 ml. of 80% ethanol.

water. Chlorination and ethanolamine treatment were repeated twice more, with water extractions after each pair.

(iii) Six 7 min. treatments with ethanolic NaOH were followed by washings with ethanol, ethanolic H_2SO_4, aqueous H_2SO_4, and again with ethanol, aqueous and sulphite extracts being then prepared.

(iv) Two 7 min. pretreatments with ethanolic NaOH were followed by six alternations of chlorination and ethanolamine treatment (concentration as in (ii)), the residue then being further treated as in (iii).

(v) As in (iv), except that four 7 min. pretreatments were given.

Finally, there is obvious interest in the action of ethanolic NaOH (4 g./100 ml.) on crude cellulose itself, as also in the solvent action of aqueous N-NaOH on the cellulose. Accordingly, samples of chlorination cellulose were submitted to the treatments shown below.

(vi) Two 7 min. pretreatments with ethanolic NaOH, the residue being washed and extracted with water and sulphite as in (iii), and

(vii) Two extractions, each for 20 min. at room temperature with 50 ml. portions of aqueous N-NaOH.

Results appear in Table 6. The extraction residues were not further chlorinated and extracted.

Table 5. *Pretreatment with ethanolic* NaOH*, *with or without subsequent treatment involving chlorination, and its influence on the extraction of furfuraldehyde-yielding materials*

(Pretreatment periods as shown; chlorination and ethanolamine treatments 7 min. each)

Final washing	Ethanol, ethanolic H_2SO_4		Ethanol, ethanolic H_2SO_4, aqueous H_2SO_4		
Pretreatment periods ...	4 × 1 hr.	1 hr.	6 × 7 min.	2 × 7 min.	4 × 7 min.
No. each of chlorinations and ethanolamine treatments ...	0	3	0	6	6
Wood residue (%)	—	—	88·69	69·71	66·54
Crude cellulose (%)	54·57	56·07	56·99	58·79	57·86
Furfuraldehyde from:					
(*a*) Wood residue (%)	—	—	14·23	13·60	12·74
(b_1) Aqueous extract (%)	2·40	2·38	2·09	1·64	2·07
(b_2) Aqueous extract (%)	0·85	0·85	—	—	—
(b_3) Aqueous extract (%)	0·63	0·67	—	—	—
(b_4) Aqueous extract (%)	0·39	0·46	—	—	—
(*c*) Sulphite extract (%)	—	—	0·67	0·96	0·89
(*d*) Crude cellulose (%)	8·17	8·54	9·68	10·12	9·21
Σb or ($b_1 + c$)	4·27	4·36	2·76	2·60	2·96
[(Σb) + *d*] or ($b_1 + c + d$)	12·44	12·80	12·44	12·72	12·17

(Results as percentage of original dry wood.)

* 4 g. of NaOH/100 ml. of 80% ethanol.

Table 6. *Extraction of furfuraldehyde-yielding material from crude cellulose*

	I*	II*
Untreated cellulose (%)	57·79	57·79
Residual cellulose (%)	54·83	45·68
Furfuraldehyde from:		
(*x*) Untreated cellulose (%)	9·18	9·18
(*y*) Residual cellulose (%)	8·09	2·56
NaOH extract (*x* − *y*)	—	6·62
(*b*) Aqueous extract (%)	0·71	—
(*c*) Sulphite extract (%)	0·31	—
(*b* + *c*)	1·02	—

(Results as percentage of original dry wood.)
* I. Pretreatment was with ethanolic NaOH (2 × 7 min.); the extracting agents were water and sulphite solution. II. No pretreatment was given; extracting agent was aqueous NaOH.

DISCUSSION

It is clear that each of the treatments investigated succeeds in rendering soluble in water a proportion of the hemicelluloses present in the original wood, a further portion—of magnitude often influenced by the type of final washing—usually being soluble in 1 % (w/v) sulphite solution. Nevertheless, the condition of complete extraction of encrusting hemicelluloses in water-soluble form without influence on the yield and composition of the subsequently prepared crude cellulose is in no case fulfilled. Analysis of the original wood indicates a furfuraldehyde yield of 5·24 % from encrusting hemicelluloses (as tentatively defined in the introduction), as will be seen from the 'no-treatment' column of Table 1 (*a*–*d*). From aqueous extracts, this figure is attained only after six alternations of chlorination and treatment with ethanolic NaOH (4 g./100 ml.); if (*b* + *c*) in the tables is held to represent furfuraldehyde from encrusting hemicellulose, then the figure of 5·24 % is substantially attained with six, eight, and ten NH_3 treatments and with four by NaOH, whilst it is exceeded with six of the latter (Tables 1 and 2). However, in all these cases the cellulose yields are definitely below the standard figure of 57·79 %, and the furfuraldehyde from cellulose correspondingly falls short of 9·18 %, the figure from cellulose prepared directly. It is worth noting that, with this type of treatment, a roughly linear relationship holds for cellulose yield and furfuraldehyde from cellulose, when both are calculated to the original wood. It is clear, then, that the chlorination-resistant material is contributing soluble hemicellulose to the extracts, and it may well prove that a definite fraction is responsible for this contribution, yielding material of substantially constant composition in amounts dependent on the intensity of the alkaline treatment. That cellulosan itself is affected by ethanolic alkali is clear from Table 6. Table 1 shows further that hemicellulose solubility increases with increasing numbers of treatments, though the indications are that NH_3 treatment never gives all the encrusting fraction in water-soluble form.

The summation (*b* + *c* + *d*) in Tables 1–5 should give 14·42 %; that the observed figures fall short of this is due to two causes. If extraction from the wood residue is incomplete, furfuraldehyde will be lost in the preparation of crude cellulose; this calls for no further comment. However, loss also occurs in the treatments leading to the production of the wood residue itself; this loss (14·42—*a*) can only be due to material passing into solution in the aqueous chlorination reagent and in aqueous H_2SO_4 where this is used for final washing. In Table 7, the losses during the preparation of wood residue are summed with (*b* + *c* + *d*) from Table 1; the extent to which the summation figure falls short of 100 % of the total furfuraldehyde reflects the loss during the final cellulose preparation. Since the furfuraldehyde determinations on sulphite extracts are necessarily made in presence of NaCl, the values for *c* are slightly in error (cf. Preece, 1940), but insufficiently to affect the present argument. It is clear that in all the cases of Table 7 considerably more furfuraldehyde-yielding material passes into solution than corresponds with encrusting hemicelluloses. Figures are available (unquoted) showing that ethanolamine is little better than NH_3 or NaOH in this respect. Increase of ethanol concentration to 95% gives no improvement with NH_3, reduction of NaOH concentration to 1 g./100 ml. merely gives reduced ex-

Table 7. *Loss of furfuraldehyde during final preparation of crude cellulose*

Alkaline reagent	NH_3*			NaOH†	
No. each of chlorinations and alkaline treatments ...	6	8	10	4	6
Furfuraldehyde as percentage of original dry wood:					
Losses from above treatments (%)	0·47	0·92	1·01	1·47	1·34
(*b* + *c* + *d*) (Table 1) (%)	12·98	13·25	13·29	12·14	12·38
Summation as percentage of total furfuraldehyde:					
Accounted for above (%)	93	98	99	94	95
Loss during crude cellulose preparation (%)	7	2	1	6	5
Chlorinations necessary in preparing crude cellulose	3	2	2	2	1

* 10 ml. of ammonium hydroxide (sp. gr. 0·88) and 90 ml. of 80 % ethanol.
† 4 g. of NaOH/100 ml. of 80 % ethanol.

traction, whilst the results for 3 and 5 g. of ethanolamine/100 ml. are substantially identical. There is thus no clear-cut distinction between two classes of hemicellulose with regard to the ease with which they can be rendered soluble in water.

Omission of chlorination gives a different type of extraction. Treatment with ethanolic NaOH (4 g./100 ml.) under a variety of conditions renders soluble in water and sulphite material yielding furfuraldehyde which, in the most favourable case (24 hr. treatment, Table 3), is equivalent to approx. 60 % of the encrusting hemicellulose. However, this material is almost certainly contaminated with chlorination-resistant material. Furthermore, both the cellulose yields and compositions are apparently anomalous; yields greater than the standard 57·79 % are frequent (see also Tables 4 and 5), whilst the furfuraldehyde yields from cellulose are consistently much greater than would be expected from the standard figures and from a consideration of the figures of Tables 1 and 2. The explanation is plain; in preparing the cellulose from the extracted wood residues, removal of lignin does not run parallel with removal of hemicellulose. Hence, working to the end-point for lignin does not necessarily ensure complete removal of encrusting hemicellulose. There is, however, a comparatively ready removal of a hemicellulose fraction of low furfuraldehyde yield. The high furfuraldehyde yields and the uncertainty of the end-point are accordingly due to relative persistence of a hemicellulose fraction under conditions which have accelerated removal of lignin. Untreated wood requires 5–6 each of chlorinations and sulphite extractions to remove lignin, as indicated by disappearance of the characteristic colour reaction; wood pretreated for as little as ½ hr. with ethanolic NaOH requires only four of each. At the other end of the scale, much hemicellulose can be removed with but little removal of lignin. Thus, 1 hr. pretreatment with ethanolic NaOH (4 g./100 ml.) renders the equivalent of some 50 % of the encrusting hemicellulose (as measured by furfuraldehyde) soluble in water or sulphite; the lignin removal at this stage may be approx. 3·87 % (i.e. 94·92–91·05 %) of the wood. It is apparent that part of the hemicellulose lends itself to ready extraction; part of the remainder is slowly converted to soluble form by the more concentrated NaOH (Table 3), though this does not apparently happen with the more dilute (Table 4), where a limit of extraction is quickly reached. Initial speed of action and subsequent retardation also appear in Table 1.

The results of Table 5 lend general support to these conclusions. It may be additionally noted that removal of hemicellulose after pretreatment with ethanolic NaOH permits a further substantial removal after subsequent alkaline treatment, so that four 1 hr. treatments give considerably greater extraction than a single 24 hr. treatment. Also, the simple alkaline treatment is just as effective as alternation of chlorination and ethanolamine treatment, though extraction by the two methods probably does not follow the same course, as is evidenced by the yield and composition of the celluloses.

The conversion of hemicelluloses into a water-soluble condition would accordingly appear to depend primarily on two factors, depolymerization (or disaggregation) and liberation from union with lignin; with some portions of the hemicellulose material both factors are involved, but the former seems to be the more generally important. The varying effects of alkalis of different types and concentrations may depend largely on their influence on molecular size, and there is little doubt that the partial dissolution of chlorination-resistant material is also governed by this factor. However, it requires also to be borne in mind that fractions which are water-soluble in the form of their sodium salts are not necessarily so when converted to the free acid; fractions which have lost water-solubility by washing with acid are nevertheless soluble in 1 % (w/v) sulphite solution, doubtless as a result of double decomposition. It may be remarked that ammonium oxalate can also restore lost water-solubility.

It is clear then that these extraction methods do not rigidly differentiate encrusting and cellulosan hemicelluloses as previously defined; it becomes doubtful whether even the normal chlorination process for production of crude cellulose separates two well-defined types with complete efficiency, though close approximation may be obtained. Nevertheless, recognition of the two groups is probably of utility as a basis for further study along botanical and constitutional lines. In the latter connexion it may be recalled that the xylan of esparto cellulose (presumably wholly or mainly cellulosan material) was stated by Hampton, Haworth & Hirst (1929) to be structurally closely related to cellulose itself, though reason was later given for some modification of this view (Haworth, Hirst & Oliver, 1934); the plant gums, on the other hand, definitely possess branched-chain structures (see, e.g. Hirst, 1942), and it may well be that the encrusting hemicelluloses are analogous to these. Whether this be true or not, it is desirable to transfer definition of the two hemicellulose groups away from dependence on the chlorination technique; ultimate decision on structure must clearly await more precise methods of extraction.

SUMMARY

1. Rigid differentiation of encrusting and cellulosan hemicelluloses is not possible by a variety of methods described.

2. Both fractions show graded ease with which they can be rendered soluble in aqueous solvents, considerable overlapping existing around the end-point of chlorination cellulose preparation.

3. Tentative distinction of two hemicellulose types seems to remain of value as a working hypothesis for further botanical and constitutional investigations.

4. Factors affecting the water-solubility of hemicellulose fractions are discussed; there is only partial parallelism between hemicellulose solubility and degree of delignification.

REFERENCES

Hampton, H. A., Haworth, W. N. & Hirst, E. L. (1929). *J. chem. Soc.* p. 1739.
Haworth, W. N., Hirst, E. L. & Oliver, E. (1934). *J. chem. Soc.* p. 1917.
Hirst, E. L. (1942). *J. chem. Soc.* p. 70.
Mitchell, R. L. & Ritter, G. J. (1940). *J. Amer. chem. Soc.* **62**, 1958.
Norman, A. G. (1936). *Biochem. J.* **28**, 2054.
—— (1937). *The Biochemistry of Cellulose, Polyuronides, Lignin, etc.* Oxford: University Press.
—— & Jenkins, S. H. (1933). *Biochem. J.* **27**, 818.
—— & Shrikhande, J. G. (1935). *Biochem. J.* **29**, 2259.
Preece, I. A. (1940). *Biochem. J.* **34**, 251.
—— (1941). *Biochem. J.* **35**, 659.

Large-scale Extraction of Protein Samples Reasonably Representative of the Whole Proteins in the Leaves of some Plants: the Amide, Tyrosine, Tryptophan, Cystine (plus Cysteine) and Methionine Contents of the Preparations

By J. W. H. LUGG and R. A. WELLER, *Nutrition Laboratory of the Division of Animal Health and Nutrition, Commonwealth Council for Scientific and Industrial Research at the University of Adelaide, Adelaide, South Australia*

(*Received* 14 *August* 1944)

Limited experimental evidence supports the statement (Lugg, 1943) that the whole proteins of the somatic, photosynthesizing, gametophyte tissues of bryophytes, and sporophyte tissues of pteridophytes and spermatophytes, appear to be of similar composition. The evidence, though meagre, was held to justify the view that for the purpose of animal nutrition studies, comprehensive analyses of pasture proteins might well be confined in the first instance to those of only a few species belonging to the most important families of pasture plants.

Considerable data already exist concerning the amide, tyrosine, tryptophan, cyst(e)ine (cystine plus cysteine) and methionine contents of the whole-leaf proteins of several spermatophytes, and of numerous protein preparations which, in most instances, may not have been properly representative of the whole protein in the leaves from which they were obtained (Lugg, 1938 *b*, *c*, 1939, 1943; Chibnall, 1939). The arginine, histidine and lysine contents of various preparations of uncertain representativeness have been estimated (Tristram, 1939), and in some instances the amounts of aspartic and glutamic acid have also been determined (Chibnall, 1939). The proline content of one such preparation has been estimated (Miller, 1936), and the glycine, alanine, valine, leucine (plus isoleucine and norleucine) and phenylalanine contents of another preparation have been estimated only very approximately and uncertainly by Lugg (quoted by Chibnall, 1939, and by Lugg, 1941).

The presence of cellulose, polyuronides and similar contaminants in protein preparations introduces no apparent systematic errors in the estimation of amide, tyrosine, tryptophan, cyst(e)ine and methionine contents when suitable, tested methods are employed. The methods available for the estimation of other amino-acids, however, are known to be subject to systematic errors or are of uncertain reliability, under these conditions. For the most satisfactory estimation of these other amino-acids, it is therefore necessary to work with preparations which are freed as far as possible from such contaminants, and if the estimations are intended to refer to the whole proteins of the leaves, it is necessary also to ensure that the preparations are properly representative of these.

The principles of methods designed to provide representative preparations of reasonable purity have been discussed elsewhere (Lugg, 1939). In the present article there will be described the extraction of large samples of such protein from the leaves of

four pasture species of great importance in Australian agriculture. Partial amino-acid compositions were determined and were used in testing representativeness.

EXPERIMENTAL

The plant materials

The plant species selected were: *Phalaris tuberosa* L. (*Phalaris*, Toowoomba canary grass), *Hordeum murinum* L.* (barley grass, wall-barley grass, mouse-barley grass), *Medicago sativa* L. (lucerne, alfalfa) and *Medicago denticulata* Willd. (burr medic†). The first two were chosen to represent the family Gramineae, and the second two the family Leguminosae. The first and third are perennials and are normally cultivated in Australian agricultural practice, whereas the second and fourth are annuals and are not usually cultivated but grow 'wild'. None of the plants is native to Australia. They are believed to have had their origins in Europe or Asia.

The plant materials were harvested from plants growing in soil which had been kept well watered and had been lightly manured with ammonium sulphate and 'super' (essentially a mixture of calcium sulphate and primary phosphate). The leaves of *M. denticulata*, with attached petioles and some short lengths of stalk, were harvested from plants which had never been cut back. Two-thirds of the material came from fairly young plants growing in seed boxes, and the rest from older plants growing in a garden. A few of the young plants had begun to flower, whilst among the older plants there were some flowers and a few burrs. Flower and burr materials were rigorously excluded. Leaves of the other species were harvested from plants which had been cut back at about the time the manures were applied and then allowed to grow for several weeks. The petioles and some short lengths of stalk were left attached to the *M. sativa* leaves. The average lengths of the *P. tuberosa* and *H. murinum* leaves were about 15 cm. in each case.

The materials were thus neither very immature nor senescent, and manuring had been light. Previous work has shown that the composition of the whole leaf proteins of a species probably varies but little under these conditions.

* Referred to as '*Hordeum leporinum* Link' in *Council for Scientific and Industrial Research Bulletin*, no. 156 (1942).

† Referred to as 'toothed medic' by Black (1922–9).

Procedure for extraction of protein

The freshly harvested material was macerated at room temperature (about 20°) with about 2·5 times its weight of a pH 9·2 borate buffer containing 1·1 g. B/l. It was chopped and torn (Lugg, 1939) rather than crushed during its single passage through a power-driven mincing machine. After breaking the surface froth with a few drops of octanol, the mash was poured on to a sieve (holes 1 mm. sq.) with continual agitation, to remove most of the fibrous material and some cell debris. The maceration was not fine, and microscopic examination of the fibrous residues revealed many unruptured cells.

The resulting green, opaque juice was adjusted carefully with NaOH to pH 9·2, and a cold (0–5°) mixture of 0·8 vol. ethanol and 0·2 vol. ether was added slowly with vigorous stirring. After about 15 min. the liquid was poured through muslin to remove most of the remaining cell debris. The bulk of the 'disintegrated granule residues' (Lugg, 1939) and some other impurities were then removed by passing the liquid through a centrifugal cream separator, running continuously except for stoppages to clean the residues from the centrifuge bowl. The liquid was submitted for about 1 min. to a field intensity of approx. $5000 \times g$. The emergent liquid was pale green and somewhat opalescent, whereas centrifugates obtained after 30 min. at $25{,}000 \times g$, and filtrates passing through paper-pulp, were found by Lugg (1939) to be almost transparent. However, large quantities of material could be treated rapidly by the cream separator.

By addition of acetic acid the liquid was brought to a pH corresponding with that of a pH 4·5 aqueous acetate buffer to which had been added 0·8 vol. ethanol, and it was then heated to about 70°. The coagulum was filtered or centrifuged from the liquid, washed, and then extracted with boiling ethanol, dilute citric acid, ethanol again and finally warm ether (Lugg, 1938*b*), to give the 'extracted' protein preparation. 'Whole' and 'residual' protein preparations were made as described by Lugg (1939); the former from a sample of the dried leaf material and the latter from a sample of the leaf residues (to which had been added the residues from the muslin filter and the centrifuge).

Data concerning the extractions are shown in Table 1. In the order of the columns are: the label of the preparation (experiment number followed by W, E or R, signifying respectively 'whole', 'extracted' or 'residual'); plant species and date;

Table 1. *Extraction of protein from the plant leaves*

Preparation	Source and date	% dry wt.	% N in dry leaf			% extracted N			% N in preparation	% ash in preparation	Dry wt. of E (g.)
			Total	Coagulable	Protein	Total	Coagulable	Protein			
1W	*Phalaris tuberosa* 9. x. 41	13·8	4·71	3·94	3·75				6·05	5·6	
1E						46·1	44·4	45·5	14·90	1·8	209
1R									2·88	6·1	
2W	*Hordeum murinum* 14. ix. 43	14·3	4·90	3·94	3·78				6·27	4·6	
2E						57·1	53·5	54·9	14·10	2·8	216
2R									3·05	6·1	
3W	*Medicago sativa* 17. ii. 41	13·7	5·07	4·00	3·88				6·78	2·0	
3E						56·2	50·5	51·1	14·15	2·3	222
3R									4·01	3·4	
4W	*Medicago denticulata* 24. ix. 41	13·1	4·91	3·84	3·75				6·87	2·7	
4E						38·0	32·3	31·4	14·55	1·4	133
4R									4·45	3·5	

percentage dry weight of the leaf material; the percentage of total N (t), coagulable N (c) and protein N (p) in the dry leaf; the percentage of the leaf N (t, c and p) appearing in the centrifuged liquid from the cream separator; the percentage of N in the dry protein preparation; and the percentage of ash in it after ignition in air at about 600°. The terms 'coagulable nitrogen' and 'protein nitrogen' have been explained fully in earlier articles (e.g. Lugg, 1939). Briefly 'protein N' means N not removed by successive extractions with boiling ethanol, dilute citric acid, ethanol and ether, from any material containing 'coagulable N'; and 'coagulable N' (in the present work) means N not removed from a material containing 'total N' by extraction at about 70° with a mixture of dilute acetic acid and 0·8 vol. of ethanol at a pH corresponding with that of an aqueous acetate buffer at pH 4·5 to which 0·8 vol. of ethanol had been added.

Analytical work

Nitrogen estimations were made by the Kjeldahl method, with selenium as catalyst and digestion at the boiling-point in the presence of considerable $NaHSO_4$ for at least 8 hr. (see Chibnall, Rees & Williams, 1943) after the digest had cleared.

The amide, tyrosine, tryptophan, cyst(e)ine and methionine contents of the protein preparations were estimated by the methods described by Lugg (1937, 1938 *a*, *b*, *c*). Some of the total S determinations, required in one of these methods, were made as described by Lugg & Weller (1944) (peroxide fusion, after alkali fusion under reducing conditions).

lated values: N (0·5, affecting all the other errors), amide (1·5), tyrosine (2), tryptophan (3), cyst(e)ine, by differential oxidation (4), methionine, by differential oxidation (5), the sum of the last two values (2), $SO_4^=$-S (5 for the higher, to 100 for the lower values), cyst(e)ine, by titration of HI digests (3), and methionine, by titration of HI digests (4).

DISCUSSION

Only the values for amide, tyrosine, tryptophan, cyst(e)ine (differential oxidation method, d.o.), methionine (d.o.), and the sum of the last two (which has a smaller random percentage error than either), may be used for demonstrating differences or similarities in the protein moieties of the preparations. The cyst(e)ine and methionine values by HI titration, being subject to systematic errors in these instances, cannot be considered entirely reliable. In a general way, however, they tend to confirm the rather larger values obtained by the less specific differential oxidation method. The sulphate-S values are to be regarded as measures of some impurity.

It is considered that the differences in composition between the W, E and R protein preparations from any one material were so small, and so uncertain in view of the magnitudes of the random errors, as to support the belief (based upon the method of preparation) that the E preparations were reasonably representative of the whole proteins in the leaf

Table 2. *Partial compositions of the 'whole', 'extracted' and 'residual' protein preparations*

					By differential oxidation				By HI titration	
Preparation	N (%)	Amide-N (%)	Tyrosine-N (%)	Tryptophan-N (%)	Cyst(e)ine-N (%)	Methionine-N (%)	Cyst(e)ine + methionine-N (%)	$\frac{100(SO_4^=\text{-S})}{N}$	Cyst(e)ine-N (%)	Methionine-N (%)
1W	6·05	4·76	2·43	2·04	1·35	1·46	2·81	0·21		
1E	14·90	4·74	2·34	2·08	1·39	1·57	2·96	0·79	1·15	1·38
1R	2·88	4·75	2·52	2·07	1·49	1·53	3·02	0·52		
2W	6·27	5·17	2·33	2·09	1·35	1·38	2·73	0·21		
2E	14·10	5·08	2·31	2·02	1·34	1·42	2·76	0·63	1·14	1·25
2R	3·05	5·04	2·38	1·93	1·48	1·45	2·93	0·56		
3W	6·78	5·34	2·43	1·93	1·14	1·24	2·38	0·05		
3E	14·15	5·12	2·30	1·94	1·17	1·36	2·53	0·35	1·01	1·19
3R	4·01	5·18	2·45	1·78	1·23	1·30	2·53	0·05		
4W	6·87	5·11	2·45	1·86	1·19	1·23	2·42	0·06		
4E	14·55	5·18	2·42	1·87	1·27	1·32	2·59	0·38	1·03	1·13
4R	4·45	5·10	2·45	1·79	1·19	1·32	2·51	0·42		

The amide and amino-acid contents are expressed in Table 2 as percentages of the protein N appearing in these forms. The values, taken mostly from replicate estimations, have been corrected for such losses as the pure, free amino-acids undergo in the analytical procedures. The random errors with W and R preparations are believed to have been not more than 1·5 times those with the E preparations, which in turn are believed not to have exceeded the following (parenthesized) percentages of the tabu-

materials. E preparations of tested representativeness and of such degrees of purity (the uncontaminated proteins would probably contain about 17% N: see Chibnall (1939)), have not been made previously on a scale involving the preparation of 100–200 g. of material. These seem to be the first E preparations to have been made from the leaves of *H. murinum* and *M. denticulata*. Two earlier, small-scale preparations from *P. tuberosa* (Lugg, 1938*b*) contained 15·4 and 15·35% N, but could not

be regarded as representative of the whole-leaf proteins. Of the earlier preparations from *M. sativa* leaves, one by Osborne, Wakeman & Leavenworth (1921) contained 16·26 % N and one by Chibnall & Nolan (1924–5) contained 15·73 % N, but they were largely deficient in proteins associated with the chloroplasts and other granular protoplasmic bodies, and probably they both, more especially the former, contained degraded protein.

The degrees of extraction of the proteins were very good, in view of the coarse maceration technique used. The leaves of *M. denticulata*, being thin and pliant, are believed to have passed through the machine with less injury than the others, thus yielding less of their protein.

The partial compositions of the protein preparations are seen to be similar to those found for the W and representative E preparations from the leaves of other members of the Gramineae and Leguminosae. The impression gained earlier (Lugg, 1938 *b*, *c*) from work with preparations of uncertain representativeness, and supported by subsequent work (Lugg, 1939, 1943), that the whole-leaf proteins of the Leguminosae are probably, in general, of slightly smaller cyst(e)ine and methionine content than those of the Gramineae, has been further substantiated.

SUMMARY

1. The extraction of large samples of protein of reasonable purity from the leaves of four important species of pasture plants has been described. The procedure, which was designed to secure reasonably representative extraction, involved the liberation (presumed) of protein from the chloroplasts and possibly other granular protoplasmic bodies, with the aid of a mildly alkaline (pH 9·2) buffer in the presence of lipoid solvents (ethanol and ether). The differences in partial composition between the extracted proteins, and those in the original leaves and in the leaf residues, were so small and uncertain that the extracted proteins were considered to be reasonably representative of the whole proteins in the leaves.

2. The plant species were *Phalaris tuberosa*, *Hordeum murinum*, *Medicago sativa* and *Medicago denticulata*. The extracted protein preparations contained 14·90, 14·10, 14·15 and 14·55 % N, respectively. Of the N in these preparations there occurred respectively, 4·74, 5·04, 5·12 and 5·18 % as amide-N; 2·34, 2·31, 2·30 and 2·42 % as tyrosine-N; 2·08, 2·02, 1·94 and 1·87 % as tryptophan-N; 1·39, 1·34, 1·17 and 1·27 % as cyst(e)ine-N; and 1·57, 1·42, 1·36 and 1·32 % as methionine-N.

We wish to thank Prof. H. C. Trumble, Dr I. F. Phipps, Mr K. A. Pike and others of the staff of the Waite Agricultural Research Institute and Mr J. W. Walters for the leaf materials, and Prof. M. L. Mitchell for the use of the mincing machine. Thanks are due also to Mr D. S. Riceman and Miss C. M. Eardley for advice on agrostological and taxonomic questions.

REFERENCES

Black, J. M. (1922–9). *Flora of South Australia*. Adelaide: Government Printer.
Chibnall, A. C. (1939). *Protein Metabolism in the Plant*. New Haven: Yale University Press.
—— & Nolan, L. S. (1924–5). *J. biol. Chem.* **62**, 173.
—— Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Council for Scientific and Industrial Research Bulletin, no. 156 (1942). *Standardized Plant Names*. Melbourne: Government Printer.
Lugg, J. W. H. (1937). *Biochem. J.* **31**, 1422.
Lugg, J. W. H. (1938 *a*, *b*, *c*). *Biochem. J.* **32**, 775, 2114, 2123.
—— (1939). *Biochem. J.* **33**, 110.
—— (1941). *J. Coun. sci. industr. Res. Aust.* **14**, 209.
—— (1943). *Biochem. J.* **37**, 132.
—— & Weller, R. A. (1944). *Aust. J. exp. Biol. med. Sci.* **22**, 149.
Miller, E. J. (1936). *Biochem. J.* **30**, 273.
Osborne, T. B., Wakeman, A. J. & Leavenworth, C. S. (1921). *J. biol. Chem.* **49**, 63.
Tristram, G. R. (1939). *Biochem. J.* **33**, 1271.

The Analysis of Hair Keratin

4. A NOTE ON THE OCCURRENCE OF SERINE IN HUMAN HAIR

By J. M. R. BEVERIDGE and C. C. LUCAS, *Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada*

(*Received* 20 *September* 1944)

Vickery & Schmidt (1931) in their review make the statement that 'serine is one of the most difficult of all the amino-acids to isolate from proteins and is even yet an exceedingly rare substance'. A reagent which has been used for precipitating *l*-serine from hydrolyzed silk fibroin, viz. *p*-hydroxy-azobenzene-*p'*-sulphonic acid (Stein, Moore & Bergmann, 1941; Stein, Moore, Stamm, Chou & Bergmann, 1942),

may prove equally useful if applied to other protein hydrolysates, in which case the isolation of serine may become relatively easy.

Previous to the appearance of the publications of Stein *et al.*, a fraction of pure serine was obtained in a yield of 1·8% from a hydrolysate of human hair. It was impossible at that time to continue these studies. Subsequent work revealed that the quantity isolated formed only a small part of the total amount present, but since it was, so far as is known, the first time that serine had been obtained from human hair, the observation is reported here. Only small amounts of serine have been isolated from other keratins: sheep wool 0·1%, ram horn 1·1% (Abderhalden & Voitinovici, 1907); horse hair 0·6% (Abderhalden & Wells, 1905); goose feathers 0·4% (Abderhalden & Count, 1905); cattle horn 1·0% (Abderhalden & Heyns, 1932); wool keratin, over 2% (Martin & Synge, 1941).

EXPERIMENTAL

A portion of thoroughly washed human hair (equivalent to 112·6 g. ash- and moisture-free keratin) was refluxed for 11 hr. with 600 ml. 9N-HCl. Excess HCl was distilled off *in vacuo* and cystine was removed as the cuprous mercaptide (Lucas & Beveridge, 1940). After removal of copper from the combined filtrates with H_2S, the solution was concentrated *in vacuo* to about 350 ml. and some tyrosine which crystallized out was removed. The dicarboxylic acid fraction was precipitated with baryta and ethanol as already described (Beveridge & Lucas, 1944). After removal of inorganic ions the solution was concentrated *in vacuo*, a crop of leucine was taken off and the remaining amino-acids were converted to copper salts by repeated evaporation with copper carbonate (Town, 1928). The water-soluble, methanol-insoluble copper salts were freed from copper, the resulting solution containing 5·413 g. N. A sample containing 3·96 g. N was used for the subsequent steps. Histidine and arginine were taken out as the silver salts, lysine as the phosphotungstate, and from the filtrate all inorganic ions were removed. The resulting solution was evaporated to about 50 ml. and treated with 9 vol. of ethanol. A white, partly crystalline precipitate came down: wt. 2·272 g. (corrected for samplings, 3·11 g.); N, 12·9%. A portion (1·803 g.), recrystallized from 60% ethanol, yielded well-crystallized plates: wt. 1·147 g., corrected for sampling, 1·972 g.

The product did not exhibit optical activity, $[\alpha]_D^{21°} = 0° \pm 0·2$ (1N-HCl, $l=2$, $c=9·72$), presumably having been racemized during the many chemical procedures to which the hydrolysates had been subjected previously. The substance melted with decomposition at 243° (corrected); the accepted decomposition temperature of *dl*-serine (Fischer & Leuchs, 1902) is 246° (corrected).

Analytical data. Found: C, 34·6, 34·5; H, 6·76, 6·28; N, 13·3, 13·3%. Calc. for $C_3H_7O_3N$: C, 34·26; H, 6·72; N, 13·34%. A second recrystallization did not change the composition. The substance was undoubtedly *dl*-serine.

Further proof of the identity of this material was obtained by subjecting it to periodate oxidation (Nicolet & Shinn, 1941); the resulting formaldehyde was quantitatively isolated as the dimedon derivative (Vorlander, 1929). Synthetic *dl*-serine was subjected to the same procedure.

Dimedon-formaldehyde derivative from	Yield (%)	m.p. (corrected)
Isolated serine	96·6	190–191°
Synthetic serine	92·5	190–191°

Mixed m.p., 190–191°; quoted m.p., 189°.

Final proof that the fraction was indeed serine was obtained by subjecting it and a sample of authentic *dl*-serine to chloramine-T oxidation. The 2:4-dinitrophenylosazones of the resulting oxidation products were prepared as described by Damodaran & Ramachandran (1941).

2:4-Dinitrophenylosazone of glyoxal derived from	Yield (%)	m.p. (corrected)
Isolated serine	77·7	325°
Synthetic serine	73·1	324°

Mixed m.p., 324°; quoted m.p., 319°; m.p. of osazone from authentic glyoxal, 323°.

A determination performed later on a portion of the same sample of hair by the procedure of Nicolet & Shinn (1941) led to a value of 7·95%. This figure includes any formaldehyde derived from hydroxylysine (if present) and may therefore be an over-estimate of the actual serine content of human hair.

The work reported here is, as far as we can ascertain, the first isolation of serine from a protein hydrolysate by means of the copper salt method of fractionation.

SUMMARY

1. Serine has been isolated from a hydrolysate of human hair.

2. Application of the periodate oxidation procedure indicates that considerably more serine is present than was isolated.

REFERENCES

Abderhalden, E. & Count, E. R. (1905). *Hoppe-Seyl. Z.* **46**, 40.
—— & Heyns, K. (1932). *Hoppe-Seyl. Z.* **206**, 137.
—— & Voitinovici, A. (1907). *Hoppe-Seyl. Z.* **52**, 348.
—— & Wells, H. G. (1905). *Hoppe-Seyl. Z.* **46**, 31.
Beveridge, J. M. R. & Lucas, C. C. (1944). *Biochem. J.* **38**, 88.
Damodaran, M. & Ramachandran, B. V. (1941). *Biochem. J.* **35**, 122.
Fischer, E. & Leuchs, H. (1902). *Ber. dtsch. chem. Ges.* **35**, 3787.
Lucas, C. C. & Beveridge, J. M. R. (1940). *Biochem. J.* **34**, 1356.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 294.
Nicolet, B. H. & Shinn, L. A. (1941). *J. biol. Chem.* **139**, 687.
Stein, W. H., Moore, S. & Bergmann, M. (1941). *J. biol. Chem.* **139**, 481.
—— —— Stamm, G., Chou, C-Y. & Bergmann, M. (1942). *J. biol. Chem.* **143**, 121.
Town, B. W. (1928). *Biochem. J.* **22**, 1083.
Vickery, H. B. & Schmidt, C. L. A. (1931). *Chem. Rev.* **9**, 169.
Vorlander, D. (1929). *Z. anal. Chem.* **77**, 241.

Substrates for Hyaluronidase

By J. MADINAVEITIA (Beit Memorial Fellow) and M. STACEY, *Chemistry Department, University of Manchester, and the Chemistry Department, University of Birmingham*

(*Received* 19 *May* 1944)

The substrate used in most of the quantitative work on the enzyme complex known as 'hyaluronidase' has been the hyaluronic acid obtained from vitreous humour, synovial fluid or umbilical cord (for example see Meyer, Smyth & Dawson, 1939; Chain & Duthie, 1940; Madinaveitia & Quibell, 1940; McClean & Hale, 1941). Hyaluronic acid occurs in many animal tissues and in some bacteria (for literature see Duran-Reynals, 1942) as a mucopolysaccharide, the carbohydrate residue of which is built up of *N*-acetyl glucosamine units and glucuronic acid units (Meyer, 1938). The action of hyaluronidase preparations is not sharply specific and is not limited to the breakdown of hyaluronic acid only. Thus, Meyer and collaborators (Meyer & Chaffee, 1940*a*, *b*; 1941) found that some preparations could hydrolyze a sulphated mucopolysaccharide from the cornea and also a sulphated polysaccharide from skin. In the latter case a liberation of sulphate residues was detected, while with both substrates hydrolysis proceeded much more slowly than with hyaluronic acid.

RESULTS

In the present investigation a number of polysaccharides, containing no detectable bound protein material and containing known constituents and in some cases known linkages, have been used as possible substrates for a preparation of hyaluronidase obtained from bovine testicle. This preparation was essentially free from β-glucosaminidase (East, Madinaveitia & Todd, 1941). Since hyaluronidase appears to have the three effects mentioned later and since two of these may eventually be correlated with polysaccharide structure, it was our object to determine whether the enzyme had (*a*) a depolymerase action on the protein-free polysaccharides, and (*b*) whether there was any hydrolytic breakdown of known linkages. Only the results of the investigation (*a*) are reported in this paper, and they are given in Table 1.

It has been found that a sulphated polysaccharide, chondroitin sulphate, from the cartilage of bovine nasal septa, is also attacked by this hyaluronidase, albeit at a slower rate than is hyaluronic acid (Table 1). In addition to its possessing sulphate residues, the cartilage polysaccharide differs from hyaluronic acid in respect of its amino-sugar constituent, which is *N*-acetyl chondrosamine. It has been suggested (Lythgoe & Madinaveitia, 1943) that hyaluronidase plays a role in making available for nutritional purposes the glucosamine present in hyaluronic acid. The above findings indicate that it can also make available the chondrosamine of some polysaccharides. In this connexion it is of interest to note that chondrosamine can be deaminated and oxidized by an enzyme system, discovered by Lutwak-Mann (1941), which attacks glucosamine.

Not every polysaccharide constituted of amino-sugars and uronic acid residues is attacked by hyaluronidase. This is exemplified in the cases of heparin and the Type I *Pneumococcus* specific polysaccharide, which are quite unaffected by the enzyme (Table 1). Of the other substrates examined, hyaluronidase attacked relatively rapidly a polysaccharide prepared from bovine placenta and more slowly an acid polysaccharide from ovarian tumour mucin, both substances being constituted of *N*-acetyl glucosamine and glucuronic acid residues and both having nitrogen content and specific rotation of the same order as those of hyaluronic acid.

A structural examination of carbohydrates of the hyaluronic acid type has not yet been carried out although some progress has been made in determining the configuration of a derivative of chondroitin sulphate (Bray, Gregory & Stacey, 1944). This material, termed 'degraded chondroitin', possesses a complicated branched chain structure having one-half of its glucuronic acid residues as 'end-groups' attached by glucosidic linkages to the rest of the molecule. These high polymers appear to possess complex structures in which several types of linkage exist and this fact is in accordance with the mode of action of hyaluronidase. We and others (Duran-Reynals, 1942) have recognized that *in vitro* three distinct phases in the breakdown of hyaluronic acid can be demonstrated. Initially there is a destruction of the power of the viscous mucopolysaccharide to form a mucin clot and we regard this as indicating that the carbohydrate has become dissociated from its combination with protein, a combination which may be to some extent ionic. Secondly, there is a dramatic decrease in viscosity of aqueous solutions of the residual complex without

Table 1. *Properties of substrates for hyaluronidase*

	Source of polysaccharide substrate																	
	Umbilical cord (hyaluronic acid)	Umbilical cord (hyaluronic acid)	Umbilical cord (degraded polysaccharide)	Cartilage (bovine trachea)	Cartilage (bovine nasal septa)	Bovine placenta	Ovarian tumour	Pancreas (galactan)	Gastric mucin	Commercial peptone	Ovomucoid	Dextran from *Leuconostoc dextranicum*	*Rhizobium radicicolum*	Luteose from *Penicillium luteum* Zukal	*Azotobacter chroococcum*	Levan from *Bacillus megatherium*	Type I *Pneumococcus*	Heparin	
No. of specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	2
Amino-sugar content (%)	35	38	35	*30	*30	34	35	15	35	35	70	—	—	—	—	—	25–35	16	3
Uronic acid content (%)	45	46	45	55	55	40	37	—	—	—	—	—	20	—	5	—	52·5	42·5	4
Other sugars	—	—	—	*Chondrosamine	*Chondrosamine	—	—	Galactose	Mannose Galactose	Mannose Galactose	Mannose Galactose	Glucose	Glucose	Glucose	Glucose	Fructose	—	—	5
Concentration (mg./ml.)	29·7	23·1	32·7	26·6	24·9	22·5	46·9	21·3	61·0	34·7	46·7	9·0	5·6	14·8	8·7	20·1	14·1	20·5	6
Specific viscosity	1·59	1·60	0·29	0·16	0·67	1·00	0·82	0·33	0·33	0·12	0·20	0·87	1·63	0·13	1·87	0·10	5·4	1·10	7
Time of incubation (min.)	Incubation at 25° with diffusing factor. Specific viscosity																		
2	—	—	0·16	0·19	—	—	—	0·44	0·30	0·14	0·19	0·95	—	0·11	—	0·11	—	—	8
3	0·77	1·32	—	—	0·66	—	0·85	—	—	—	—	—	—	—	—	—	—	—	
4	—	—	—	—	—	0·98	—	—	—	—	—	—	1·73	—	1·73	—	—	—	
5	—	—	0·12	0·18	—	—	—	—	0·28	0·13	0·20	—	—	0·08	—	0·11	—	—	
6	0·52	—	—	—	0·62	—	—	0·42	—	—	—	—	—	—	—	—	—	—	
8	—	—	0·11	0·18	—	0·89	0·80	—	—	—	—	0·93	—	0·08	—	0·11	—	—	
9	0·41	1·00	—	—	—	—	—	—	0·28	0·13	0·20	—	—	—	1·66	—	—	—	
10	—	—	—	—	0·60	—	—	0·36	—	—	—	—	1·63	—	—	—	—	—	
11	—	—	—	—	—	—	—	—	—	—	—	—	—	0·08	—	—	—	—	
12	—	—	—	—	—	—	0·78	—	—	—	—	0·94	—	—	—	—	—	—	
13	—	—	—	—	—	—	—	—	0·27	—	—	—	—	—	—	—	—	—	
14	—	0·92	—	—	—	0·83	—	0·36	—	—	—	—	—	—	—	—	—	—	
15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·64	—	—	—	
16	—	—	—	—	0·57	—	—	—	—	—	—	—	1·61	—	—	—	—	—	
18	—	0·82	—	—	—	—	—	—	0·27	—	—	0·95	—	—	—	—	—	—	
21	—	—	—	—	0·55	—	—	—	—	—	—	—	—	—	—	—	—	—	
22	—	—	—	—	—	0·76	0·74	—	—	—	—	—	1·62	—	—	—	—	—	
23	—	0·74	—	—	—	—	—	—	0·27	—	—	—	—	—	—	—	—	—	
28	—	0·67	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
31	—	—	—	—	0·52	—	—	—	—	—	—	—	—	—	—	—	—	—	
32	—	—	—	—	—	—	0·70	—	—	—	—	—	—	—	—	—	5·20	1·00	
Action of enzyme	+	+	+	−	+	+	+	−	±	−	−	−	−	−	−	−	−	−	
	Incubation for 18 hr. at 37°. Specific viscosity																		
Without diffusing factor	1·37	1·51	0·25	0·16	0·63	0·87	0·79	0·31	0·31	0·11	0·16	0·80	1·61	0·05	1·50	0·17	5·4	1·11	9
With diffusing factor	0·08	0·09	0·06	0·15	0·07	0·42	0·16	0·32	0·22	0·11	0·16	0·85	1·56	0·08	1·56	0·11	5·25	1·08	

liberation of reducing sugars and this means that the enzyme is exerting a powerful 'polysaccharide depolymerase' action. Finally, there is a slower progressive liberation of reducing sugars, namely glucuronic acid and *N*-acetyl glucosamine, which indicates a hydrolytic breakdown of the polysaccharide chain.

Polysaccharides which were not attacked by the preparation were the following: (*a*) the blood group A polysaccharides from both gastric mucin and from pepsin, the latter containing units of *l*-fucose, mannose, galactose and *N*-acetyl glucosamine (Stacey, 1943); (*b*) a galactan from the pancreas (Stacey, 1944); (*c*) ovomucoid, containing units of *N*-acetyl glucosamine, mannose and galactose united in a complicated branched chain structure (Stacey & Woolley, 1940); (*d*) dextran from *L. dextranicum* constituted of long chains of glucose units (Peat, Schlüchterer & Stacey, 1939) linked through the 1- and 6-positions; (*e*) the *Rhizobium radicicolum* polysaccharide (Cooper, Daker & Stacey, 1938) containing units of glucuronic acid and glucose linked in a manner generally similar to the Type III *Pneumococcus* polysaccharide; (*f*) luteose from *P. luteum* Zukal (Anderson, Haworth, Raistrick & Stacey, 1939), a 'β-dextran' constituted solely of units of glucose linked through the 1- and 6-positions; (*g*) an *Azotobacter* polysaccharide containing units of glucuronic acid and glucose (Cooper *et al.* 1938); (*h*) the *B. megatherium* levan which is built up from units of fructose linked through the 2- and 6-positions (Cooper & Preston, 1935; Lyne, Peat & Stacey, 1940).

A method is described in the experimental section for preparing hyaluronic acid free from detectable protein.

EXPERIMENTAL

Determination of hyaluronidase activity

Aqueous solutions of the polysaccharides having the concentration indicated in § 6 of the table were mixed with 0·2 vol. of a mixture of equal amounts of M-NaCl and M-citrate buffer pH 4·5 and were filtered through glass wool. In Table 1 (§ 7) the specific viscosity of each of these solutions (at 25°) diluted with 0·2 vol. of water is recorded.

The enzyme used was a solution of a preparation of hyaluronidase from bovine testicle purified by lead acetate and ammonium sulphate precipitation (Madinaveitia & Quibell, 1940; East *et al.* 1941). Buffered polysaccharide solution (1 vol.) and enzyme (0·2 vol.) were mixed at 25° and variation of the viscosity with time at this temperature followed, as described by Madinaveitia & Quibell (1940). Control solutions without enzyme and the solutions with enzyme were placed for 18 hr. in a thermostat at 37°. Toluene was added as a preservative. In some solutions a slight precipitate was formed; any precipitate was removed in the centrifuge and the specific viscosity determined at 25°. The prolonged incubation at 37° permits the establishment of the significance of small variations of viscosity observed during the shorter incubation at 25°.

Preparation of substrates

In the preparation of the polysaccharides, emphasis has been laid on the removal from them (as far as present methods permit) of detectable 'bound' protein constituents and on subsequent isolation of an essentially homogeneous polysaccharide fraction as far as can be shown by fractionation methods. It is realized that some degradation of carbohydrate structure may have occurred and that the macromolecular state may not be the optimum obtainable for each polysaccharide, for it has been emphasized by one of us (Stacey, 1943) that the nature of a carbohydrate prepared from a polysaccharide-protein complex varies sharply with the mode of isolation. Where an effect of the enzyme has been observed we consider that it is an effect on the polysaccharide structure. Tests for proteins were carried out on 2% solutions of the polysaccharides, using, for example, the following reagents at various concentrations: lead acetate, sulphosalicylic acid, trichloroacetic acid, Esbach's picric acid reagent, Millon's solution, glycocylic acid, and phosphotungstic acid. The following tests were also negative: biuret, Sakaguchi.

The amino sugars were estimated by the method of Elson & Morgan (1933) and the uronic acid content by gravimetric estimation of CO_2 evolved on heating the polysaccharides with 12% HCl saturated with NaCl.

(1) *Hyaluronic acid* (cf. McClean & Hale, 1941). Umbilical cords were collected and stored under 1% aqueous phenol, in which they could be kept for several months. They were freed from blood-clots by dissection, washed with a rapid stream of water, and minced coarsely. This material (500 g.) was stirred briskly into boiling water (1 l.) and immediately filtered through a fine-meshed sieve. Four or five extractions were made in this way, the last extract being relatively non-viscous. The combined extracts were cooled and acidified with 5N-acetic acid, when a mucin clot separated. The mucin clot was collected on a cloth and was dissolved in a small amount of N-NaOH. The solution was diluted to 300 ml. and the pH adjusted to 8·5 by addition of acetic acid. $BaCO_3$ (20 g.) was added and the liquid boiled briskly for a few minutes until a little of the cleared supernatant failed to give a precipitate with acetic acid. $BaCO_3$, with coagulated protein material, was removed and washed on the centrifuge, and hyaluronic acid (Ba salt) obtained from the clear supernatant by addition of 2 vol. of ethanol. The product, a fibrous precipitate, was washed with ethanol and ether and dried (5 g.). It was purified by dissolving it in water, centrifuging and precipitating with ethanol and by dialysis followed by ethanolic precipitation, until all the fractions showed $[\alpha]_D^{20°} - 60°$ in water. Yield, 3–4 g. (found: N, 5·0; amino sugar, 35·4; uronic acid, 45%). No tests for proteins were given by, for example, 2% solutions of this and other polysaccharides described below or by the products obtained from them on acid hydrolysis. The product gives highly viscous solutions in water and does not give a precipitate on addition of acetic acid. We regard this fact as a further indication of the absence of protein since the power to give a precipitate can be restored by addition of a serum (0·1 ml.) to a 1% aqueous solution (10 ml.) of hyaluronic acid or its salts. The ability to give a precipitate with acetic acid appears to be retained even when the hyaluronic acid has been degraded by alkali treatment, but with degraded hyaluronic acid the precipitate obtained on acidification is not a true mucin clot. When hyaluronic acid is combined with protein it is found that

the protein-hyaluronic acid complex becomes completely insoluble in water on intensive drying at 100° or on being kept over P_2O_5 for some weeks. In this respect the complex differs obviously from our preparation of hyaluronic acid.

(2) Prepared from sample (1) by shaking a dilute aqueous solution of it with carbon tetrachloride to remove possible traces of extraneous protein, followed by centrifuging and ethanolic precipitation. It showed $[\alpha]_D^{20°} - 58°$ in water (found: N, 5·0; amino sugar, 38·0; uronic acid, 46%).

(3) Prepared from sample (2) by heating its aqueous solution at pH 8·5 for 2 hr. and then isolating the product by ethanolic precipitation. It showed $[\alpha]_D^{20°} - 40°$ in water (found: N, 4·8; amino sugar, 35; uronic acid, 45%).

(4) This specimen was prepared from bovine tracheal cartilage by essentially the method described by Bray *et al.* (1944) and was carefully fractionated by precipitation from aqueous solution by ethanol. The main fraction showed $[\alpha]_D^{17°} - 26°$ in water (found: N, 3·0; S, 2·3; amino sugar, 30·0; uronic acid, 55·2; K (K-salt), 18·5; acetyl, 8·4%) and could not be further separated into fractions having different properties. Neither the polysaccharide nor its acid hydrolysis products gave any of the usual tests for amino acids or proteins.

(5) This sample was prepared from bovine nasal septa by the same method as specimen (4). The main product free from detectable protein had $[\alpha]_D^{17°} - 25°$ in water (found: N, 4·3; S, 2·5; amino sugar, 30·0; uronic acid, 55·0; K (K salt), 18·4; acetyl, 8·3%). This chondroitin sulphate gave aqueous solutions which were more viscous than those obtained from the tracheal preparations, but evidence has not yet been provided to show that chondroitin sulphates from all types of cartilage are identical.

(6) Bovine placental tissue was separated by dissection, and dissolved rapidly in warm dilute NaOH. The solution was immediately neutralized with acetic acid and stirred at 70° with $BaCO_3$ for 10 min. The filtered solution was poured into ethanol and the precipitate carefully purified by fractional precipitation from aqueous solution by ethanol. A product (50% of the crude product) was obtained; it was free from protein and could not be separated into fractions showing different properties. After dialysis through cellophan the polysaccharide had $[\alpha]_D^{20°} - 45°$ in water (found: N, 4·5; amino sugar, 34·2; uronic acid, 40·0%).

(7) Part of a large non-malignant ovarian tumour was minced coarsely and the cystic fluid obtained by passing the mince through cloth. The fluid was diluted with an equal volume of 2% sodium acetate and the solution rapidly raised to the boiling-point in the presence of $BaCO_3$. The solution, filtered whilst hot, was cooled and poured into excess ethanol and the copious precipitate of polysaccharide material collected (centrifuge). It was separated by ethanol precipitation into four main fractions:

F_1: yield, 10%; $[\alpha]_D^{20°} - 50°$; ash, 25%.
F_2: yield, 50%; $[\alpha]_D^{20°} - 40°$; ash, 20%.
F_3: yield, 25%; $[\alpha]_D^{20°} - 11°$; ash, 40%.
F_4: yield, 10%; $[\alpha]_D^{20°} - 12°$; ash, 35%.

Fraction F_2 was the only one further examined. It was dialyzed for 12 hr. in parchment and then isolated by concentration of the solution followed by precipitation with acetone. Extraneous protein material was removed by shaking a solution of the polysaccharide with small quantities of carbon tetrachloride until no more insoluble material was thrown out of solution.

The polysaccharide was now precipitated by fractional addition of ethanol and the fractionation continued until an essentially homogeneous fraction (7% yield of crude product) was obtained. This had the following properties (Ba salt): $[\alpha]_D^{17°} - 40°$ in water (found: N, 4·5; amino sugar, 34·5; uronic acid, 37·4%).

(8) Commercial trypsin was dissolved in water (5% solution); 10% trichloroacetic acid was added until no further precipitate was formed. This precipitate was removed (centrifuge) and discarded. The supernatant liquid was dialyzed through cellophan for 12 hr., concentrated *in vacuo* and a precipitate thrown out in three fractions by adding ethanol. The middle fraction (yield, 40%; $[\alpha]_D^{17°} - 30°$) was dialyzed in cellophan for 24 hr., the solution concentrated *in vacuo* and a polysaccharide precipitated by adding acetone (3 vol.). The polysaccharide was further fractioned until an essentially homogeneous product (15% yield) was obtained. This showed $[\alpha]_D^{17°} - 35°$ in water (found: N, 2·8; amino sugar, 15·5; galactose (estimated by isolation of mucic acid), *c.* 50%).

(9) Method of Landsteiner & Harte (1940). It showed $[\alpha]_D^{20°} - 10°$ in water (found: N, 4·8; amino sugar, 35·0%). This product still contained amino-acid residues in agreement with Landsteiner & Harte (1940).

(10) Method of Landsteiner & Harte (1940). It showed $[\alpha]_D^{21°} + 8°$ in water (found: N, 5·0; amino sugar, 35·0%) and still contained amino-acid residues.

Samples (11)–(16) inclusive are adequately described in the literature.

(11) Method of Stacey & Woolley (1940). It showed $[\alpha]_D^{21°} - 57°$ in water.

(12) Method described by Stacey & Youd (1938). It showed $[\alpha]_D^{20°} + 180°$ in water.

(13) Method of Cooper *et al.* (1938). It showed $[\alpha]_D^{21°} - 20°$ in water.

(14) Method of Anderson *et al.* (1939). It showed $[\alpha]_D^{20°} - 38°$ in water.

(15) Method of Cooper *et al.* (1938). It showed $[\alpha]_D^{20°} - 10°$ in water.

(16) Method of Cooper & Preston (1935). It showed $[\alpha]_D^{20°} - 43°$ in water.

(17) *Type I* Pneumococcus *polysaccharide*. This product was prepared under the personal direction of Dr M. Heidelberger, and was freed from protein by Sevag's method (1934). It had $[\alpha]_D^{20°} + 235°$, uronic acid 52·5, N 5·2, and acetyl 4·5%. Amino-sugar determinations were unsatisfactory (25–35%).

(18) *Heparin*. A sample of heparin (British Drug Houses Ltd., 500 units/mg.) (0·5 g.) was purified by Sevag's method (1934) followed by dialysis and ethanolic fractionation methods. The product used (0·35 g.) had $[\alpha]_D^{20°} - 42°$ in water (found: S, 9·5; N, 2·5; uronic acid, 42·5; amino sugar, 16%) and contained no detectable protein constituents.

SUMMARY

1. The action of hyaluronidase in destroying the viscosity of aqueous solutions of various polysaccharides has been investigated.

2. In addition to being active against hyaluronic acid and closely related polysaccharides from placenta and tumour mucin, it lowers the viscosity of chondroitin sulphate from nasal septa.

REFERENCES

Anderson, C. G., Haworth, W. N., Raistrick, H. & Stacey, M. (1939). *Biochem. J.* **33**, 272.
Bray, H. G., Gregory, J. & Stacey, M. (1944). *Biochem. J.* **38**, 142.
Chain, E. & Duthie, E. S. (1940). *Brit. J. exp. Path.* **21**, 324.
Cooper, E. A., Daker, W. D. & Stacey, M. (1938). *Biochem. J.* **32**, 1752.
—— & Preston, J. F. (1935). *Biochem. J.* **24**, 2267.
Duran-Reynals, F. (1942). *Bact. Rev.* **6**, 197.
East, M. E., Madinaveitia, J. & Todd, A. R. (1941). *Biochem. J.* **35**, 872.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Landsteiner, K. & Harte, R. A. (1940). *J. exp. Med.* **71**, 551.
Lutwak-Mann, C. (1941). *Biochem. J.* **35**, 610.
Lyne, R. R., Peat, S. & Stacey, M. (1940). *J. chem. Soc.* p. 237.
Lythgoe, B. & Madinaveitia, J. (1943). *Biochem. J.* **37**, 6.
McClean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
Madinaveitia, J. & Quibell, T. H. H. (1940). *Biochem. J.* **34**, 625.
Meyer, K. (1938). *Cold Spr. Harb. Symp. Quant. Biol.* **6**, 91.
—— & Chaffee, E. (1940*a*). *Amer. J. Ophthal.* **23**, 1320.
—— —— (1940*b*). *Proc. Soc. exp. Biol., N.Y.*, **43**, 487.
—— —— (1941). *J. biol. Chem.* **138**, 491.
—— Smyth, E. M. & Dawson, M. H. (1939). *J. biol. Chem.* **128**, 319.
Peat, S., Schlüchterer, E. & Stacey, M. (1939). *J. chem. Soc.* p. 581.
Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
Stacey, M. (1943). *J. Soc. chem. Ind., Lond.*, **62**, 110.
—— (1944). Unpublished observations.
—— & Woolley, J. M. (1940). *J. chem. Soc.* p. 184.
—— & Youd, R. (1938). *Biochem. J.* **32**, 1943.

The Synthesis of Cysteine-(Cystine-) Tyrosine Peptides and the Action Thereon of Crystalline Pepsin

By C. R. HARINGTON and ROSALIND V. PITT RIVERS, *National Institute for Medical Research, Hampstead, N.W.* 3

(*Received* 19 *August* 1944)

In connexion with some projected immunochemical work it became desirable to have peptides containing both cystine and tyrosine. Since such compounds, none of which has hitherto been available, might also be expected to be of some intrinsic interest, the syntheses of cysteyl- and cystyltyrosine and of tyrosylcysteine and -cystine were undertaken and are here described.

The paper also contains an account of the action of crystalline pepsin on these dipeptides and their acylated derivatives, the study of which has led to results of unexpected interest.

Syntheses of l(−)*cysteyl*-l(−)*tyrosine and* l(−)*tyrosyl*-l(−)*cysteine*

The syntheses have followed conventional lines and call for little comment. It was found necessary to work throughout with the *S*-benzyl derivative of cysteine and to remove the *S*-benzyl group at the final stage.

Cysteyltyrosine. The *N*-carbobenzyloxy derivative of *S*-benzylcysteine ethyl ester was converted through the hydrazide into the azide; the latter was coupled in ethereal solution with *O*-benzoyltyrosine ethyl ester. The resulting ethyl ester of *S*-benzyl-*N*-carbobenzyloxycysteyl-*O*-benzoyltyrosine on treatment with alkali in the cold gave *S*-benzyl-*N*-carbobenzyloxycysteyltyrosine from which the desired peptide was obtained by reduction with sodium in liquid ammonia.

Tyrosylcysteine. *N*-Carbobenzyloxytyrosyl azide was coupled with *S*-benzylcysteine ethyl ester and the product was hydrolyzed with alkali to give *N*-carbobenzyloxy-*S*-benzylcysteine; sodium and liquid ammonia were again used to remove carbobenzyloxy and benzyl groups, yielding tyrosylcysteine.

The yields throughout both syntheses were good. The peptides were both readily converted into the corresponding cystine derivatives by aeration in slightly alkaline solution, the cystine peptides thus obtained being more soluble in water than either of the component amino-acids.

Both the cysteine peptides crystallized easily as did also tyrosylcystine; cystyltyrosine had first to be converted into the well-characterized hydrochloride from which the peptide could be recovered in crystalline form. It may be noted that tyrosylcystine and cystyltyrosine appear to represent the first crystalline cystine peptides to be recorded, although crystalline peptides of cysteine have been obtained by other workers.

The *N*-carbobenzyloxy derivatives of the peptides required for the enzymic experiments described below were prepared by reaction of the cystine peptides with benzylchloroformate, the corresponding

N-carbobenzyloxy cysteyl peptides being obtained by reduction with zinc dust and sulphuric acid.

Action of pepsin on acylated and non-acylated cysteine-(cystine-) tyrosine peptides

The action of crystalline pepsin was studied on (*a*) the *N*-carbobenzyloxy derivatives of cysteyl- and cystyltyrosine and of tyrosylcysteine and -cystine and (*b*) on the corresponding free peptides, the experiments being carried out both at pH 4·0 and at pH 1·8.

The results are summarized in Table 1, from which the following facts emerge: (1) cysteine-(cystine-) tyrosine peptides afford good examples of simple synthetic substrates for peptic action; (2) the action of pepsin on these substrates is much more marked at pH 4·0 than at pH 1·8 which is the commonly accepted optimum for the hydrolysis of proteins by pepsin; (3) although the *N*-carbobenzyloxy derivatives are the most readily hydrolyzed, the action of pepsin extends in this series to the free peptides, which thus represent the first true peptides shown to be attacked by this enzyme; (4) all the substrates, whether acylated or not, are more susceptible to the action of pepsin when they are in the reduced form. These points may be discussed in relation to the work of Bergmann on the nature of the action of pepsin.

(1) Hitherto the only synthetic substrates shown to be attacked by pepsin have been a series of derivatives of peptides containing tyrosine or phenylalanine and glutamic acid in which the amino group of the aromatic amino-acid was combined with the α-carboxyl group of an acylated glutamic acid (Fruton & Bergmann, 1939).

(2) Our substrates behave similarly to those of Fruton & Bergmann in that their hydrolysis by pepsin is much more rapid at pH 4·0 than at pH 1·8, although in the case of cysteyltyrosine and its *N*-carbobenzyloxy derivative we have observed a significant degree of splitting at the latter pH.

(3) As has been noted, the substrates employed by Fruton & Bergmann were all acylated (*N*-carbobenzyloxy-) peptides, with the one exception of glycylglutamyltyrosine; the free glutamyl peptides of tyrosine and phenylalanine were not attacked at all; moreover, it was necessary that the second carboxyl of the glutamic acid residue should be free, since the corresponding glutaminyl derivatives were much more resistant to peptic hydrolysis. In discussing the matter Bergmann & Fruton (1941) lay considerable emphasis on these points, concluding (*a*) that the hydrolysis of a peptide linkage by pepsin is conditional on the absence of a free amino group from the immediate neighbourhood of the linkage attacked and (*b*) that substrates for peptic action must contain more than one carboxyl group.

It is difficult to see how these conclusions can stand in the light of the results which we now record. As to (*a*), reference to Table 1 shows that, whilst acylation of the free amino groups of our peptides undoubtedly favours peptic hydrolysis, the degrees of splitting observed with some of the free peptides are by no means insignificant; moreover, in one case (cysteyltyrosine) splitting is observed at pH 1·8, a reaction at which the free amino group will be charged. In respect to (*b*) it is only necessary to note that none of our reduced substrates, which are the most readily split, has more than one free carboxyl group, and that the rate of hydrolysis of one of them (*N*-carbobenzyloxycysteyltyrosine) is comparable with that of the most sensitive substrate used by Fruton & Bergmann (1939), namely *N*-carbobenzyloxyglutamyltyrosine.

(4) The observation that our substrates are attacked by pepsin much more rapidly in the reduced (—SH) than in the oxidized (—SS—) form

Table 1. *Percentage splitting of acylated and non-acylated cysteine-(cystine-) tyrosine peptides by crystalline pepsin at* 38°

	pH 4·0			pH 1·8	
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.
A. *Acylated peptides:*					
N-Carbobenzyloxytyrosylcysteine	22	39	46	0	10
N-Carbobenzyloxytyrosylcystine	6	7	11	0	0
N-Carbobenzyloxycysteyltyrosine	33	53	85	23	32
N-Carbobenzyloxycystyltyrosine	9	21	29	0	6
N-Carbobenzyloxy-*S*-benzylcysteyltyrosine	—	26	30	—	—
B. *Non-acylated peptides:*					
Tyrosylcysteine	—	22	29	0	8
Tyrosylcystine	—	5	8	0	8
Cysteyltyrosine	—	31	55*	13	21
Cystyltyrosine	—	5	7	0	0

* In a larger experiment cysteyltyrosine (570 mg.) was hydrolyzed with pepsin at pH 4·0 for 60 hr. On removal of pepsin and concentration of the solution crystalline material separated; when recrystallized this gave 119 mg. of pure tyrosine, i.e. 30% of the theoretical yield for complete splitting of the peptide.

appears to raise a point of considerable interest. It had already been shown by Fruton & Bergmann (1939) that the action of crystalline pepsin on *N*-carbobenzyloxyglutamyltyrosine was unaffected by the presence of cysteine. We have ourselves performed the additional control experiment of incubating the twice crystallized pepsin employed by us in the presence of cysteine on the one hand and cystine on the other; under these conditions we have found that at a given pH the 'pepsin blank', i.e. the amount of free amino-nitrogen contained in the original pepsin together with any that may be formed by autolysis of the latter is, within the limits of error, the same whether cysteine or cystine has been present; we are therefore satisfied that the apparently greater susceptibility of the —SH peptides to peptic action is a genuine phenomenon.

This being accepted, it is of interest to recall (*a*) that pepsin is commonly recognized to act much more vigorously on denatured than on native proteins and (*b*) that denaturation of proteins is accompanied by the appearance of —SH groups. The possible interrelation of these facts with our observation is evident; taking into account also the fact that free tyrosine can be liberated from proteins by pepsin it seems not unlikely that at least one point of attack of a denatured protein by pepsin may be a cysteyltyrosine or tyrosylcysteine linkage.

A point of minor interest which appears from the results recorded in Table 1 is that throughout the whole series and at both hydrogen-ion concentrations the cysteyltyrosine derivatives are more sensitive to peptic action than those of tyrosylcysteine. It may also be noted that the rate of splitting of *S*-benzyl-*N*-carbobenzyloxycysteyltyrosine is similar to that of *N*-carbobenzyloxycystyltyrosine and much less than that of the corresponding cysteyl compound; this again emphasizes the importance of the —SH group for the reaction.

EXPERIMENTAL

A. *Synthesis of cysteyltyrosine*

S-Benzylcysteine ethyl ester hydrochloride. *S*-Benzylcysteine (Wood & du Vigneaud, 1939) (4·4 g.) was suspended in ethanol (60 ml.) and the mixture saturated with a vigorous stream of hydrogen chloride; the clear solution which resulted was cooled, resaturated with hydrogen chloride and evaporated under diminished pressure. The crystalline residue was taken up in hot ethanol and the solution mixed with dry ether; the material which separated on cooling was collected and a further small crop was obtained by evaporating the mother liquor, dissolving the residue in the minimum of ethanol and precipitating with ether. The total yield was 4·72 g. (96 %).

The compound formed plates from ethanol-ether, m.p. 156–157°. (Found: N, 5·3; Cl, 12·9 %. $C_{12}H_{18}O_2NSCl$ requires N, 5·1; Cl, 12·9 %.)

S-Benzyl-N-carbobenzyloxycysteine ester. *S*-Benzylcysteine ethyl ester hydrochloride (13·77 g.) was suspended in chloroform (125 ml.); the suspension was cooled in ice-salt and shaken vigorously with 2N-sodium hydroxide (25 ml.); benzyl chloroformate (5·5 ml.) was then added with shaking. This was followed by 2N-sodium carbonate (25 ml.) and a further 5·5 ml. of benzyl chloroformate, vigorous shaking and intermittent cooling being maintained. The chloroform solution was separated, washed twice with dilute hydrochloric acid, twice with aqueous sodium bicarbonate and twice with water; it was dried and evaporated and the residue was redissolved in chloroform; addition of light petroleum caused separation of the ester, which was collected and dried. The yield was 15·65 g. (84 %).

The ester crystallized from chloroform-light petroleum in needles, m.p. 52–53°. (Found: N, 3·7; S, 8·0 %. $C_{20}H_{23}O_4NS$ requires N, 3·8; S, 8·6 %.)

S-Benzyl-N-carbobenzyloxycysteyl hydrazide. The above ester (25·5 g.) was boiled under reflux for 3·5 hr. with ethanol (60 ml.) and hydrazine hydrate (6 ml.). On cooling, a first crop of 15·3 g. separated. A further crop was obtained by concentrating the mother liquor and leaving in an unevacuated desiccator overnight with sulphuric acid to remove excess of hydrazine. The total yield was 19·7 g. (80 %). The hydrazide crystallized from ethanol in large prisms, m.p. 133–134°. (Found: N, 11·8 %. $C_{18}H_{21}O_3N_3S$ requires N, 11·7 %.)

O-Benzoyl-N-carbobenzyloxytyrosine ethyl ester. *N*-Carbobenzyloxytyrosine ethyl ester (11·5 g.) was dissolved in pyridine (50 ml.) and treated with benzoyl chloride (8 ml.). The solution was warmed for 15 min. on the boiling water-bath, cooled and poured into 250 ml. of 3N-hydrochloric acid; the oil which precipitated rapidly hardened. After keeping overnight in the ice-chest, the product was collected, triturated with dilute hydrochloric acid, filtered off, washed with water and dried. It was crystallized from a mixture of benzene and ligroin from which it separated in prisms, m.p. 92°. The yield was 14 g. (94 %). (Found: N, 3·1 %. $C_{26}H_{25}O_6N$ requires N, 3·1 %.)

O-Benzoyltyrosine ethyl ester hydrochloride. The above ester (6·9 g.) was dissolved in methanol (80 ml.) and hydrochloric acid (~~1·25~~ 2·5 ml. of 6·85N) and shaken in an atmosphere of hydrogen with palladium black (1·08 g.); evolution of carbon dioxide was complete in 1¾ hr. The filtered solution was evaporated in a vacuum, the evaporation being repeated after addition of ethanol; the residue was taken up in hot ethanol and the solution mixed with dry ether. The total yield (two successive crops) was 4·56 g. (85 %).

The compound crystallized from ethanol-ether in rectangular prisms, m.p. 225° (decomp.). (Found: N, 4·0; Cl, 10·15 %. $C_{18}H_{20}O_4NCl$ requires N, 4·0; Cl, 10·15 %.)

S-Benzyl-N-carbobenzyloxycysteyl-O-benzoyltyrosine ethyl ester. *S*-Benzyl-*N*-carbobenzyloxycysteyl hydrazide (6·3 g.) was dissolved in a mixture of water (79 ml.), acetic acid (79 ml.) and 3N-hydrochloric acid (11·7 ml.); the cooled solution was treated gradually, with stirring, with sodium nitrite (1·23 g.) in water (13 ml.). The azide which separated was extracted with ether (200 ml.) and the extract, after rapid drying over sodium sulphate, was added immediately to a solution of *O*-benzoyltyrosine ester in ether; the latter solution was prepared by shaking a chilled suspension of *O*-benzoyltyrosine ester hydrochloride (8·4 g.) in ether (200 ml.) with N-sodium hydroxide (24 ml.), separating the ether and drying over sodium sulphate. Crystallization of the reaction product began immediately after mixing of the ethereal solutions; after 40 hr. in the ice-chest the crystals were collected, washed with cold ether and dried. The yield was 7·96 g. (71 % calculated on the hydrazide) of a product crystallizing from ethanol in spear-shaped needles, m.p. 149°. (Found: N, 4·4; S, 4·8 %. $C_{36}H_{36}O_7N_2S$ requires N, 4·4; S, 5·0 %.)

S-Benzyl-N-carbobenzyloxycysteyltyrosine. The above ester (12 g.) was dissolved in dioxan (80 ml.) and shaken mechanically for 1 hr. with 5N-sodium hydroxide (11·3 ml.); the mixture was diluted with water, acidified and the product collected, dried and extracted with boiling ligroin to remove benzoic acid. It was crystallized from ethanol diluted with a little water and separated in sheaves of fine needles, m.p. 198–200°. The yield was 8·7 g. (92 %). (Found: N, 5·5 %. $C_{27}H_{28}O_6N_2S$ requires N, 5·5 %.)

Cysteyltyrosine. *S*-Benzyl-*N*-carbobenzyloxycysteyltyrosine (5·4 g.) was dissolved in liquid ammonia (75 ml.); sodium in small pieces was added to the stirred solution until a blue colour was obtained, 1·3 g. being required. Ammonium sulphate (3·75 g.) was introduced to discharge the blue colour and the ammonia was evaporated as rapidly as possible; removal of ammonia was completed by warming at 40° in a vacuum followed by 3½ hr. in a vacuum desiccator over sulphuric acid. The residue was taken up in 0·5N-sulphuric acid (80 ml.) and filtered from dibenzyl; it was then treated with mercuric sulphate (Hopkins's reagent as modified by Kendall, McKenzie & Mason, 1929) until precipitation was complete, 16·5 ml. being needed. Next day the precipitate was collected; it was washed five times by grinding with water and refiltration on a Buchner funnel and was then decomposed by suspension in water (80 ml.) and saturation with hydrogen sulphide; the mixture was filtered and the precipitate again suspended in water, saturated with hydrogen sulphide and filtered. The combined filtrates were concentrated to a low bulk under diminished pressure (bath temperature not > 50°), transferred to a dish and adjusted to pH about 4·8 by addition of concentrated ammonia. Crystals were obtained by adding ethanol cautiously to a small portion of the solution and on inoculation of the main bulk crystallization rapidly set in. After keeping overnight the precipitate was collected and dried (1·6 g.). A further crop (0·41 g.) was obtained by concentrating the mother liquor and again adjusting the pH by addition of sodium acetate, the total yield thus being 70 %.

The dipeptide crystallized from water, in which it is readily soluble at the boiling-point, in prisms having m.p. > 300° (decomp.). It had $[\alpha]_D^{25°} + 15·2°$ ($c = 5$ in N-hydrochloric acid). (Found: C, 50·2; H, 5·7; N, 10·0; S, 11·3 %. $C_{12}H_{16}O_4N_2S$ requires C, 50·7; H, 5·6; N, 9·9; S, 11·3 %.)

In 50 % ethanol containing N-hydrochloric acid 28·4 mg. required 9·95 ml. 0·01N-iodine (calc. 10·0 ml.) (cf. Lucas & King, 1932).

Cystyltyrosine. Cysteyltyrosine (2 g.) was suspended in water (50 ml.) and cold saturated baryta was added until all was in solution and the pH was about 8·5; a trace of ferrous sulphate was added and a current of air was drawn through the solution. When the nitroprusside reaction had become negative the solution was freed from barium with sulphuric acid and concentrated under diminished pressure, being finally evaporated to complete dryness in a desiccator over sulphuric acid. The residue was taken up in a little water and left in a partly evacuated desiccator over a dish of ethanol; after 2 days the peptide had separated in a hard crust which was slightly yellow in tint and was not crystalline; it was collected, washed with ethanol and dried; the yield was 1·65 g. (80 %). The solution of the peptide in N-hydrochloric acid at about 5 % concentration slowly deposited crystals; addition of an equal volume of concentrated hydrochloric acid and chilling gave a good yield of the *hydrochloride*; bunches of prismatic needles, m.p. 242–243° (decomp.). The hydrochloride was dissolved in water and treated with 1 equiv. of sodium hydroxide; the solution was evaporated to a low bulk in a vacuum desiccator and the partly crystalline residue collected. On solution in a small amount of warm water with the minimum hydrochloric acid and neutralization to about pH 4·0 with sodium acetate the peptide was obtained in the form of rosettes of spear-shaped needles, m.p. 294° (decomp.). It gave no nitroprusside reaction and reduced no iodine in acid solution. It had $[\alpha]_D^{25°} - 50·8°$ ($c = 5$ in N-hydrochloric acid). (Found: N, 9·9; S, 10·9 %. $C_{24}H_{30}O_8N_4S_2$ requires N, 9·9; S, 11·3 %.)

N-Carbobenzyloxycystyltyrosine. Cystyltyrosine (0·564 g.) was dissolved in water (5 ml.) with sodium carbonate (0·52 ml. of 3·86 N; 1 equiv.); the solution was chilled and shaken with benzyl chloroformate (0·35 ml.); a second equal quantity of sodium carbonate was added and shaking continued until the reaction was over. During the reaction the sparingly soluble sodium salt of the product separated. The mixture was acidified with hydrochloric acid (3 ml. of 3 N) and extracted with ethyl acetate; the extract was washed with dilute hydrochloric acid and then three times with brine; it was dried over sodium sulphate and evaporated under diminished pressure to a low volume. The product was isolated by precipitation from the concentrated ethyl acetate solution with light petroleum, but could not be obtained crystalline. It had m.p. 158°. (Found: N, 6·8; S, 7·4 %. $C_{40}H_{42}O_{12}N_4S_2$ requires N, 6·7; S, 7·7 %.)

N-Carbobenzyloxycysteyltyrosine. N-Carbobenzyloxycystyltyrosine (0·4 g.) was dissolved in ethanol (20 ml.); 2·1 N-sulphuric acid (10 ml.) was added to the warmed solution followed by zinc dust in excess; intermittent warming was continued until vigorous reaction ceased (about 35 min.); the solution was then filtered, diluted with water and freed from ethanol by distillation under diminished pressure. The residue was extracted with ethyl acetate and the latter was washed with dilute hydrochloric acid and water and dried; the product was isolated by evaporation of the ethyl acetate and precipitation with light petroleum; it was crystallized from aqueous ethanol, from which it separated in prisms having m.p. 160–162°. The yield was 0·27 g. (68 %). (Found: N, 6·8; S, 7·4 %. $C_{20}H_{22}O_6N_2S$ requires N, 6·7; S, 7·7 %.) In 50 % ethanol containing N-hydrochloric acid 41·8 mg. required 5·1 ml. 0·01 N-iodine (calc. 5·0 ml.).

B. *Synthesis of tyrosylcysteine*

N-Carbobenzyloxytyrosyl hydrazide. N-Carbobenzyloxytyrosine ethyl ester (8 g.) was dissolved in ethanol (12 ml.) and treated with hydrazine hydrate (1·75 ml.); the mixture was boiled under reflux for 75 min., when the contents of the flask set to a semi-solid mass. After chilling, the solid was collected with the aid of more ethanol and dried. A further small crop was obtained by concentrating the mother liquor. The total yield was 5·73 g. (75 %). For analysis the hydrazide was recrystallized from ethanol, in which it is sparingly soluble; it formed needles having m.p. 220–221°. (Found: N, 12·7 %. $C_{17}H_{19}O_4N_3$ requires N, 12·8 %.)

N-Carbobenzyloxytyrosyl-S-benzylcysteine ethyl ester. This compound was prepared as in the case of *S*-benzyl-*N*-carbobenzyloxycysteyl-*O*-benzoyltyrosine ester (see above); the azide was prepared by addition of sodium nitrite (1·85 g.) in water (18 ml.) to the above hydrazide (8·65 g.) dissolved in acetic acid (64 ml.), 3 N-hydrochloric acid (45 ml.) and water (170 ml.), and the free ester was obtained from *S*-benzylcysteine ethyl ester hydrochloride (9·6 g.). Crystallization of the product started 10 min. after the ethereal solutions of azide and ester were mixed; after keeping for 40 hr. in the ice-chest the crystals were collected, washed with ether and dried. The yield was 10·82 g. (81 % calculated on the hydrazide); two-thirds of the excess *S*-benzylcysteine ester employed could be recovered as hydrochloride by saturating the ethereal mother liquor with dry hydrogen chloride.

The product of the reaction crystallized from 75 % ethanol in needles having m.p. 142–145°. (Found: N, 5·2; S, 6·2 %. $C_{29}H_{32}O_6N_2S$ requires N, 5·2; S, 6·0 %.)

N-Carbobenzyloxytyrosyl-S-benzylcysteine. The above ester (6·05 g.) was stirred with a mixture of N-sodium hydroxide (22·6 ml.) and dioxan (23 ml.) until dissolved. After 30 min. at room temperature the solution was diluted with water and made acid to Congo red with dilute hydrochloric acid; an oil separated which soon solidified. After keeping some time in the ice-chest the solid was collected and dried. The yield was quantitative. The acid crystallized from aqueous methanol in fine needles having m.p. 166°. (Found: N, 5·3 %. $C_{27}H_{28}O_6N_2S$ requires N, 5·5 %.)

Tyrosylcysteine. The reduction of *N*-carbobenzyloxytyrosyl-*S*-benzylcysteine and the isolation of the dipeptide were carried out exactly as described above for cysteyltyrosine. From 13·05 g. of starting material there were obtained 4·83 g. (66 %) of tyrosylcysteine.

The dipeptide crystallized from water, in which its solubility is similar to that of cysteyltyrosine, in large prisms melting above 300° with decomposition. It had $[\alpha]_D^{23°} + 22·6°$ ($c = 5$ in N-hydrochloric acid). (Found: C, 50·9; H, 5·5; N, 10·1; S, 11·4 %. $C_{12}H_{16}O_4N_2S$ requires C, 50·7; H, 5·6; N, 9·9; S, 11·3 %.)

In 50 % ethanol containing N-hydrochloric acid 28·4 mg. reduced 10·1 ml. 0·01 N-iodine (calc. 10·0 ml.).

Tyrosylcystine. Tyrosylcysteine (2 g.) was suspended in water (50 ml.) and brought into solution by addition of baryta (27·5 ml. of 0·35 N). The solution was aerated until the nitroprusside reaction was negative (several hours). Barium was removed exactly with sulphuric acid and the filtrate was concentrated under diminished pressure, the final stage being carried out in a vacuum desiccator over sulphuric acid. Crystalline material amounting to 1 g. separated, and a further crop of 0·48 g. of non-crystalline but almost pure product was obtained by concentrating the mother liquor and precipitating with ethanol.

The dipeptide crystallized from water in prisms, m.p. 292° (decomp.). It had $[\alpha]_D^{23°} - 70{\cdot}8°$ ($c = 5$ in N-hydrochloric acid). (Found: N, 9·9; S, 11·0%. $C_{24}H_{30}O_8N_4S_2$ requires N, 9·9; S, 11·3%.)

N-*Carbobenzyloxytyrosylcystine*. Tyrosylcystine (0·42 g.) was dissolved in water (5 ml.) with sodium carbonate (0·75 ml. of 2N) and the solution was shaken at 0° with benzylchloroformate (0·3 ml.); a further equal quantity of sodium carbonate was added and shaking continued until the reaction was over. The sodium salt which had separated was collected, dissolved in water (40 ml.) and acidified with dilute hydrochloric acid. The precipitate was collected, washed and dried. The yield was 0·45 g. (72%).

For analysis the compound was purified by dissolving in ethyl acetate, washing the solution with dilute hydrochloric acid and water, drying and precipitating with light petroleum after concentration; it was crystallized from dilute ethanol from which it separated in prisms having m.p. 150° (decomp.). (Found: N, 6·7%. $C_{40}H_{42}O_{12}N_4S_2$ requires N, 6·7%.)

N-*Carbobenzyloxytyrosylcysteine*. *N*-Carbobenzyloxytyrosylcystine (0·4 g.) was dissolved in ethanol (20 ml.) and warmed to 50°; sulphuric acid (10 ml. of 2·1N) was added followed by excess of zinc dust; intermittent warming was maintained for about 35 min., by which time the vigorous reaction was over. The filtered solution was diluted with water and freed from ethanol by distillation under diminished pressure; it was then extracted with ethyl acetate. The extract was washed with dilute hydrochloric acid and water, dried and concentrated; addition of light petroleum precipitated the product, which could not however be obtained crystalline. The yield was 0·22 g. (54%) of a substance having m.p. 120° (decomp.). (Found: N, 6·8; S, 7·3%. $C_{20}H_{22}O_6N_2S$ requires N, 6·7; S, 7·7%.)

In 50% ethanol containing N-hydrochloric acid 41·8 mg. required 4·7 ml. of 0·01N-iodine (calc. 5·0 ml.); the compound was thus 94% reduced.

Enzymic experiments

The crystalline pepsin used was prepared from 1:10,000 pepsin (Parke Davis Ltd.) by the method of Northrop (1939) and was twice crystallized. Its activity was determined by the haemoglobin method described by Northrop.

The course of the enzymic splitting of the peptides was followed by amino-acid determinations by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941), this being the only method applicable to cysteine and cystine. The conditions for satisfactory quantitative determinations of both cysteine (cystine) and tyrosine were found to be 8 min. heating at pH 1·0 with 0·1 g. of ninhydrin for a 2 ml. sample and these conditions were adhered to throughout.

The actual experiments were carried out in the reaction vessels for the ninhydrin amino-acid determinations. In the various series of experiments the contents of the vessels were as follows.

Acylated peptides at pH 4·0. 0·04 mM. of the peptide in 0·4 ml. 0·1N-sodium hydroxide was mixed with 1·0 ml. 0·2M-acetate buffer of pH 4·0 followed by 0·1 ml. 0·4N-hydrochloric acid and 0·5 ml. 4% pepsin solution.

Non-acylated peptides at pH 4·0. As above except that 0·02 mM. of peptide in 0·4 ml. 0·05N-sodium hydroxide followed by 0·1 ml. 0·2N-hydrochloric acid were used.

Acylated peptides at pH 1·8. 0·04 mM. peptide in 0·4 ml. 0·1N-sodium hydroxide was mixed with 1·0 ml. 0·08N-hydrochloric acid followed by 0·1 ml. 0·4N-hydrochloric acid and 0·5 ml. 4% pepsin solution.

Non-acylated peptides at pH 1·8. As above except that 0·02 mM. peptide in 0·4 ml. 0·05N-sodium hydroxide, 1·0 ml. 0·08N-hydrochloric acid and 0·1 ml. 0·2N-hydrochloric acid were used.

Blank determinations. The control experiments carried out by Fruton & Bergmann (1939) consisted in incubation of the substrates with pepsin which had been inactivated by bringing to pH 8·0. We have ourselves done controls of this nature but since, particularly at pH 1·8, there is the possibility of partial autolysis of the pepsin with resultant increase in the blank, it has seemed better to us to use the figures obtained by incubating active pepsin under the conditions of the experiment but in the absence of substrate; in separate determinations it was found that the substrates themselves, in absence of pepsin, gave negligible blank figures both at pH 4·0 and 1·8. The pepsin blanks determined in this way are larger than those measured by the method of Fruton & Bergmann so that their use in the calculations gives a more conservative estimate of the true hydrolysis of the substrates.

The remote possibility that the apparently greater extent of hydrolysis of the —SH substrates might be due to activation of the autolysis of pepsin was excluded by an experiment in which pepsin was incubated at pH 4·0 in presence of (*a*) 0·01M-cysteine and (*b*) 0·005M-cystine; the pepsin blanks in this experiment were indistinguishable from one another and from the blank determined in the ordinary way without substrate.

Finally the use of acetate buffer in the concentrations employed was shown in separate experiments to be without effect on the ninhydrin amino-acid determination.

SUMMARY

1. The syntheses are described of cysteyl- and cystyltyrosine, of tyrosylcysteine and -cystine and of the *N*-carbobenzyloxy derivatives of these peptides.

2. It is shown that both the *N*-carbobenzyloxy derivatives and to a smaller but significant extent the free peptides are hydrolyzed by crystalline pepsin; the hydrolysis is more rapid at pH 4·0 than at pH 1·8. The bearing of these results on Bergmann's theory of the conditions determining peptic action is discussed.

3. The cysteine derivatives are more susceptible to peptic hydrolysis than the corresponding compounds containing cystine; this observation is considered in relation to the fact that pepsin attacks proteins more rapidly after denaturation which is itself accompanied by the appearance of —SH groups.

REFERENCES

Bergmann, M. & Fruton, J. S. (1941). *Advances in Enzymology*, **1**, 63.

Fruton, J. S. & Bergmann, M. (1939). *J. biol. Chem.* **127**, 627.

Kendall, E. C., McKenzie, B. F. & Mason, H. L. (1929). *J. biol. Chem.* **84**, 657.

Lucas, C. C. & King, E. J. (1932). *Biochem. J.* **26**, 2076.

Northrop, J. H. (1939). *Crystalline Enzymes*, p. 129. New York: Columbia University Press.

Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.

Wood, J. L. & du Vigneaud, V. (1939). *J. biol. Chem.* **130**, 110.

Riboflavin Content of Canteen Meals

By MARGARET KERLY, *Department of Biochemistry, University College, London*

(*Received* 19 *August* 1944)

At a time when diet is restricted and when the public is encouraged to supplement home supplies by eating at canteens, the dietary value of the meals served is important, especially in regard to the content of essential nutrients possibly in short supply. The estimations of riboflavin reported here formed part of a survey of meals served in canteens in the Leatherhead district.

METHODS

Riboflavin was estimated microbiologically on all meals and on three the values were checked by the rat growth method.

Preparation of samples. A complete meal was collected in the canteens as served, care being taken to see that the portions of food represented average servings. The whole meal was air-dried at 70–80°, then thoroughly mixed and finely ground. Samples of the dry powder were autoclaved with dilute HCl for 15 min. at a pressure of 15 lb.; the extracts adjusted to pH 6·8 with KOH, made to volume and filtered. Usually 2 g. of powder hydrolyzed with 25 ml. 0·01 N-HCl and diluted after neutralization to 100 ml. gave an extract of suitable strength. Extraction with 0·25 N-HCl (Barton-Wright & Booth, 1943) or water also gave satisfactory extraction, but the presence of starch in the water extracts complicated subsequent estimation of riboflavin (see below); the more dilute acid was preferred for extraction, as a larger volume, and better mixing with the sample, could be used without increase in the salt content of the extract. KOH was used to adjust the pH; it is recommended by Snell & Strong (1939) for neutralization of the growth medium. In several cases where fat was removed from the dry powder by extraction with petrol ether, riboflavin determinations on the defatted powder were not significantly different from those on the unextracted powder. For the rat tests the dry food powder was fed as a supplement to the basal ration. In these experiments nine meals were bulked before drying and grinding to obtain sufficient quantities of food powder.

Microbiological method. The organism used was a strain of *Lactobacillus helveticus*, kindly given to us by Dr C. H. Gray and obtained by him originally from the National Collection of Type Cultures. Fresh inocula were prepared from the stock agar culture for each series of estimations as recommended by Barton-Wright & Booth (1943). The medium used was that described by Snell & Strong (1939). Xylose, nicotinic and pantothenic acids (Barton-Wright & Booth, 1943) or the liver extract used in the rat-growth experiments, which contained all B vitamins except riboflavin (Chattaway, Happold & Sandford, 1943), added to the medium, caused no increase in acid production. Starch added to the medium (0·4–1·0%) caused additional acid production in control tubes and at all concentrations of riboflavin, but did not alter the relationship between acid production and riboflavin content, i.e. the slope of the curve obtained by plotting acid produced against riboflavin content was not altered by the presence of starch. Similarly, alteration of the pH of the medium (pH 6·4–7·0) affected the amount of acid produced, but not the response to added riboflavin. As a routine extracts and media were adjusted to pH 6·8.

Fermentations were carried out in McCartney culture bottles having tops with a central hole and rubber liner (A. Gallenkamp and Co. Ltd., London). Inoculations were made by injecting through the rubber liner one drop of the saline suspension of the organism from a sterile syringe. Bottles were incubated for 24 hr. only because this shorter time was found to give a more nearly linear relationship between acid production and riboflavin content. Acid production was estimated by titrating directly into the bottle

with 0·1 N-NaOH to a pH of 6·8. The end-point must be estimated within 0·2 pH unit, because the medium is well buffered over the range pH 6·2–8·0. With food extracts which may be deeply coloured it is essential to use a comparator (Barton-Wright & Booth, 1943).

The riboflavin content of extracts was estimated by determining growth at four levels of concentration (each in triplicate) and comparing the slope of the response curve with that of a similar curve obtained from determinations on a standard riboflavin solution. The amount of dried food extracted was chosen so that 1 ml. extract contained approximately 0·1 μg. riboflavin. From the best straight line relating volume of NaOH required to neutralize the acid produced to volume of extract used, the extract equivalent to 1 ml. NaOH was determined. In a similar manner for each batch of extracts the riboflavin equivalent of 1 ml. of NaOH was determined for the standard riboflavin solution (0·1 μg./ml.). By equating the NaOH equivalent of extract and riboflavin solution the concentration of riboflavin in the extract was calculated. This method was adopted as it was found that food extracts gave a 'blank' value different from that of the standard riboflavin solutions, i.e. the curves when extrapolated backwards did not pass through the same zero value (0·3–0·6 ml. of NaOH) as that given by the control tubes containing no riboflavin. This acid production in the control tubes was not due to bacterial growth as similar values were obtained after sterilizing and incubating uninoculated medium. If the food extracts were photolyzed at an alkaline pH all growth-promoting activity was destroyed; such extracts after readjustment of pH, inoculation and incubation gave titration values similar to those obtained from control tubes containing no riboflavin. The curves obtained from starch-free HCl extracts of food were similar to those obtained on medium containing starch. Curves from water extracts of food had a higher 'blank', but the same slope. As satisfactory results were obtained by comparison of the slope of acid production curves for food extracts and for standard riboflavin solutions, the reason for the initial increased response with the food extracts was not further investigated.

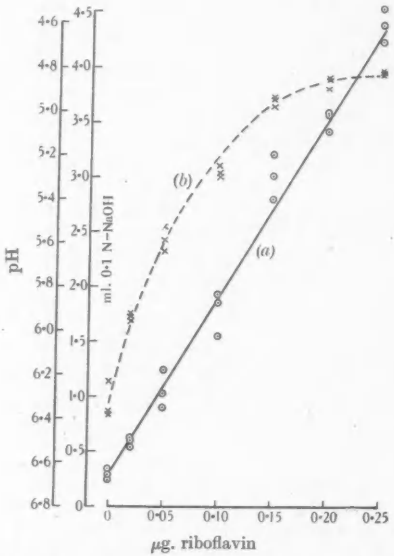


Fig. 1. Relationship between riboflavin concentration in medium and acid production measured by (*a*) titration with 0·1 N-NaOH and (*b*) change in pH of the medium.

It has been suggested (Silber & Mushett, 1942; Davis, Mawson, Newland, Kon & Thompson, 1942) that acid production may be estimated by change in pH of the culture medium. This would be preferable to titration for coloured extracts, but as a suitable meter was not available until most of this survey was completed, the method was used for one extract (coffee) only. Satisfactory results were obtained between concentrations of 0·02 and 0·15 μg. riboflavin (Fig. 1).

Rat growth method. The method was that of El Sadr, Macrae & Work (1940) and the regimen followed that described by Copping (1943), using the maize starch basal diet. Each food was fed at two levels, calculated to contain approximately 6 and 12 μg. riboflavin, to groups of four rats; two similar control groups, fed 6 and 12 μg. pure riboflavin respectively, were used for each food. Feeding was continued for 4 weeks and riboflavin content of the food powders calculated from the curves obtained by plotting growth response against the logarithm of the dose.

RESULTS

In a series of estimations of the riboflavin content of a number of food powders, results usually agreed within 10 %, but larger variations were encountered in some cases. With one meal, consisting of beef, potatoes, cabbage and treacle tart, determinations were made on the undried sample and one and two weeks later, after drying and grinding (Table 1). Since there was no consistent fall in riboflavin content it was concluded that no loss was caused by drying and storing. Sampling difficulties prevented estimation of recovery of riboflavin added to a whole meal, but recovery from a portion of porridge, treated in the same way as the whole meals, was reasonably satisfactory (Table 1).

For three meals good agreement was found between the results of the microbiological and the rat-growth methods.

No obvious correlation was observed among the dishes included in the meals and the riboflavin content. Three meals which contained cheese all gave high values, but two of them also contained egg and bread pudding.

A few estimations were made on samples of Ceylon and Indian teas and on a sample of coffee. Extraction was made by pouring boiling water on to the tea or coffee, allowing to stand for 10 min. and then filtering off the extract. Standing for 3 min. before filtration did not give complete extraction, but further standing up to 45 min. did not increase the riboflavin content of the extract. These results are of the same order as those reported by Drummond & Moran (1944).

Table 1. *Riboflavin content of canteen meals*

	Riboflavin content (mg./meal)	
	(*a*) Microbiological method	(*b*) Rat-growth method
School canteens		
Beef, potato, salad, rice pudding	0·28	—
Lentil soup, potato, watercress, steamed pudding, custard	0·29	—
Corned beef, potato, cabbage, treacle tart	0·29	—
Mutton, potato, beans, watercress, steamed pudding, custard	0·30	—
Shepherd's pie, salad, chocolate pudding	0·32	—
Beef, potato, cabbage, steamed pudding, semolina sauce	0·32	0·34
Fish pie (cod), potato, cabbage, cake pudding, sauce	0·35	—
Toad-in-the-hole, potato, cabbage, prunes, custard	0·43	—
Beef, potato, cabbage, treacle tart: (*a*) Before drying	0·40	—
(*b*) After 1 week	0·35	—
(*c*) After 2 weeks	0·43	—
Steak pie, potato, cabbage, rice pudding, prunes	0·43	0·50
Beef, potato, salad, blancmange, jam	0·55	—
Egg and cheese pie, fish pie (tinned salmon), potato, salad, bread pudding, custard	0·74	0·73
Egg and cheese pie, potato, salad, bread pudding, custard	0·92	—
Fish pie (tinned salmon), potato, salad, bread pudding, custard	0·93	—
British restaurant		
Hot pot, potato, cabbage, steamed pudding, custard	0·55	—
Cheese pudding, potato, salad, steamed syrup pudding	0·90	—
Industrial canteen		
Cold meat, potato, beetroot, jam roll, custard, bread, butter	0·40	—
Foodstuffs		
Porridge. 100 g. wet weight: (*a*) Before drying	0·017	—
(*b*) After drying	0·015	
(*c*) After addition of 0·038 mg. riboflavin and drying	0·049	—
	μg./g.	
Indian tea: Sample 1	11	
2	8	
3	11	
Ceylon tea: Sample 1	9	
2	12	
3	16	
Coffee*	1	

* Growth estimated by pH change.

SUMMARY

The riboflavin content of a number of meals served at canteens has been estimated by the microbiological technique, and the values found confirmed on three meals by the rat-growth method of estimation.

I should like to express my gratitude to Miss A. M. Copping and to Dr C. H. Gray for their advice on the rat-growth and microbiological methods respectively; my thanks are also due to the Lister Institute of Preventive Medicine for rats from their stock colony, to Mr A. L. Bacharach, of Glaxo Laboratories Ltd., for a supply of liver extract, and to Roche Products Ltd. for a supply of aneurin.

REFERENCES

Barton-Wright, E. C. & Booth, R. G. (1943). *Biochem. J.* **37**, 25.

Chattaway, F. W., Happold, F. C. & Sandford, M. (1943). *Biochem. J.* **37**, 298.

Copping, A. M. (1943). *Biochem. J.* **37**, 12.

Davis, J. G., Mawson, E. H., Newland, D. G., Kon, S. K. & Thompson, S. Y. (1942). Communication read at a Symposium on the Microbiological Assay of Vitamins of B Complex at the National Institute for Research in Dairying, Shinfield.

Drummond, J. C. & Moran, T. (1944). *Nature, Lond.*, **153**, 99.

El Sadr, M. M., Macrae, T. F. & Work, C. E. (1940). *Biochem. J.* **34**, 601.

Silber, R. H. & Mushett, C. W. (1942). *J. biol. Chem.* **146**, 271.

Snell, E. E. & Strong, F. M. (1939). *Industr. Engng Chem.* (Anal. ed.), **11**, 346.

Micro-determination of *iso*Citric and *cis*-Aconitic Acids in Biological Material

By H. A. KREBS and L. V. EGGLESTON, *Department of Biochemistry, University of Sheffield*

(*Received* 22 *August* 1944)

Tracer experiments of Wood, Werkman, Hemingway & Nier (1941) and of Evans & Slotin (1941) suggest that the order in which citrate, *iso*citrate and *cis*-aconitate arise from oxaloacetate and pyruvate in pigeon liver is:

$$\begin{matrix}\text{oxaloacetate} \\ +\text{pyruvate}\end{matrix} \rightarrow \textit{cis}\text{-aconitate} \rightleftharpoons \textit{iso}\text{citrate}$$
$$\downarrow\uparrow$$
$$\text{citrate}$$

and not, as had originally been suggested (see Krebs, 1943),

$$\begin{matrix}\text{oxaloacetate} \\ +\text{pyruvate}\end{matrix} \rightarrow \text{citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \textit{iso}\text{citrate.}$$

In order to be able to examine the new hypothesis we have worked out methods for the micro-determination of *cis*-aconitic and *iso*citric acids, a preliminary account of which has been published in the Proceedings of the Biochemical Society (Krebs & Eggleston, 1943*b*). So far no specific micro-methods for the estimation of the two acids have been described. The polarimetric method (Martius, 1938) for the determination of *iso*citric acid, based on the high rotation of the molybdate complex of the acid, is unspecific and is therefore not generally applicable. Moreover, this method requires comparatively large quantities—about 25–50 mg.—if the determination is to be reasonably accurate. The determination of *iso*citric acid by isolation of its dimethylester lactone requires even larger quantities. The micro-determination of *cis*-aconitic acid (Johnson, 1939), depending on the manometric measurement of the hydrogen required for quantitative hydrogenation, is likewise unspecific and applicable only if other unsaturated compounds are absent.

The following principle permits the determination of a few milligrams of (−)*iso*citric and *cis*-aconitic acids in biological material provided that citric acid is not present in very much larger quantities. Three portions of neutral tissue extract or tissue suspension are measured. The first is incubated with 'aconitase' whereby *iso*citric and *cis*-aconitic acids are converted into citric acid, the latter being determined according to Pucher, Vickery & Leavenworth (1934). In the second portion citric acid is determined directly. From the third portion *cis*-aconitic acid is removed by conversion into the *trans*-form; it is then treated like the first sample. The difference in the citric acid content between the first and second samples indicates the sum of (−)*iso*citric and *cis*-aconitic acids in the sample; the difference between the second and third the amount of (−)*iso*citric acid; the difference between the first and third the amount of *cis*-aconitic acid.

Thus the determination of the two acids requires three determinations of citric acid. If only one of the acids is present (apart from citric acid) two citric acid determinations are required.

I. DETERMINATION OF CITRIC ACID

The method of Pucher, Sherman & Vickery (1936) for colorimetric citric acid determination in biological material (based on the conversion of citric acid into pentabromoacetone, which yields a yellow colour on addition of sulphide) is highly specific and is applicable to small quantities (0·2–1·0 mg.) of citric acid, but has one major disadvantage: its limits of error are considerable, amounting to about 10% when the method is applied to animal tissues. We have not been able to identify with certainty the steps where the errors arise; it is probable that they are connected with the relative instability of the colour-giving compound under the conditions of the estimation. This instability is shown by the following observations: 1 mg. pentabromoacetone was dissolved in 1 ml. 4% Na_2S, diluted with 5 ml. of water and 4 ml. of dioxan, and shaken for 15 min. with 50 ml. of various organic solvents. The colour of the aqueous solution was then compared with that of a standard solution prepared at the same time, but not treated with solvents. The recovery was as follows:

Solvent	Colour recovered (%)
Light petroleum (40–60°)	51
Toluene	58
Xylene	43
Ether	45
*cyclo*Hexane	47

Purification of light petroleum or toluene with concentrated H_2SO_4, alkaline $KMnO_4$ and bromine water did not improve the recovery. Gradual fading of the colour also occurred in the absence of the above solvents, the rate of fading being affected by substances in the solution; it is increased, for example, by ethanol. Within certain limits the loss of colour is in proportion to the concentration of pentabromoacetone, but at higher concentrations the loss is relatively smaller. This seems to be the reason why the amounts of citric acid to be determined by the colorimetric method of Pucher *et al.* (1936) should not exceed 1 mg.

Because of the relatively large margin of error of the colorimetric method we have, in the present

work, chiefly used the volumetric method of Pucher *et al.* (1934), with some modifications. This method is also based on the conversion of citric acid into pentabromoacetone, but the latter is determined volumetrically by titrating the bromide set free on treatment with sulphide. The main modifications concern the following points:

(1) The oxidation and bromination are carried out in more acid (3N) solution and $KMnO_4$ is gradually, instead of suddenly, added to the solution. This was found to increase the yield of pentabromoacetone to about 97%, whilst the original procedure yielded 88%. By increasing the acidity to 6N it is possible, as shown in Table 1, to raise the yield to the theoretical level of 100%, but at the same time the method becomes less specific (see later). For this reason we prefer to use an acidity of 3N.

Table 1. *Effect of acidity on the yield of pentabromoacetone from citric acid*

5 mg. citric acid; 'revised method' refers to the procedure described in the text, with the exception of the acid concentration which was varied as stated in the first column.

Normality of H_2SO_4	No. of exps.	Yield (%) Highest	Lowest	Mean
Method of Pucher *et al.* (1934)				
1·5	2	88·6	87·7	88·2
Revised method				
1·5	3	92·6	90·9	91·8
3	2	97·6	97·5	97·6
6	5	101·4	98·2	99·9
12	1	—	—	100·0

(2) For the extraction of pentabromoacetone from light petroleum a solution of 4% Na_2S in N-NaOH was used. This reagent extracts more rapidly than the original solution, which is of importance when the amount of citric acid exceeds 10 mg.

(3) The titration of the bromide is carried out with solutions of one-fifth of the strength used by Pucher *et al.* (1934). This increases the accuracy of the titration provided that the volume of the solution is kept low. Before the titration the volume is therefore reduced to about 7 ml. No loss of halide is incurred during the process if carried out as described later.

Procedure

Deproteinization. The solution to be examined is mixed with one-fifth of its volume of 20% metaphosphoric acid and filtered after 5 min. (For the keeping qualities of HPO_3 see Krebs, Smyth & Evans, 1940.)

Removal of further interfering substances (cf. Pucher *et al.* 1936). A measured portion of the filtrate containing 0·5–20 mg. citric acid, preferably 5–10 mg., is transferred to a 500 ml. conical flask, diluted with water to 80 ml., and, after addition of 6 ml. 20N-H_2SO_4, boiled until the volume is reduced to about 35 ml. This removes certain interfering substances such as β-ketonic acids (oxaloacetic, acetoacetic, acetonedicarboxylic acids), acetaldehyde and acetone, whilst no citric acid is lost. The solution is cooled to room temperature, an excess of bromine water (usually about 3 ml.) is added, and the mixture is allowed to stand for 10 min. If a precipitate is formed (as is commonly the case with plant material) the solution is allowed to stand for a further period of 20 min., with fresh additions of bromine water if the first portion of bromine is decolorized. The precipitate is filtered off and the filter is washed with a few ml. of 3N-H_2SO_4.

Conversion into pentabromoacetone. To the solution are added 2 ml. 11·9% KBr and, drop by drop, enough 4·7% $KMnO_4$ to produce a permanent dark purple colour. During the addition of $KMnO_4$ the flask should be shaken vigorously. The solution is allowed to stand for 15 min. during which the excess of permanganate, as indicated by the purple colour, must be maintained. After the lapse of this period ferrous sulphate (20% $FeSO_4.7H_2O$ in 1% H_2SO_4) is added in 1–2 ml. quantities until the colour of the solution turns orange. Then an excess of 5 ml. ferrous sulphate is added which changes the colour to lemon. If more than 2 mg. of citric acid are present a precipitate of pentabromoacetone is usually visible.

Extraction of pentabromoacetone. The solution is transferred to a 350 ml. pear-shaped separating funnel freshly greased with 'rubber grease' and provided with a stem of at least 7 mm. internal diameter (narrower stems tend to retain fluid through capillary forces). The transfer is completed by rinsing into the separating funnel with several 5 ml. quantities of light petroleum (b.p. 40–60°). In all, 25 ml. petroleum are added and the mixture is shaken for 2 min. The aqueous layer is then drawn off and the petroleum layer (contaminated with traces of the aqueous layer) is collected in another vessel. The funnel is rinsed with 2 portions of 3–5 ml. petroleum which are added to the main extract. The aqueous layer is quantitatively washed into the funnel and shaken again with 25 ml. petroleum. The aqueous layer is drawn off and discarded. This second extract (with a trace of the aqueous layer) is combined with the first extract. The petroleum is then washed four times with 15 ml. water to remove halide and iron salts.

Extraction with sulphide. 5 ml. of 4% Na_2S in N-NaOH are measured into the funnel and shaken with the petroleum for 1 min. The aqueous phase is collected in a 200 ml. long-necked boiling flask. The shaking is repeated twice with 5 ml. of water and then again with 5 ml. alkaline sulphide and two 5 ml. portions of water. The presence of pentabromoacetone is indicated by a yellow colour of the aqueous phase. If the quantity of citric acid to be determined exceeds 20 mg. it may be necessary to make a third series of extractions with the sulphide

solution and water. Whether this step is needed is indicated by the intensity of the yellow colour of the second sulphide portion.

Dehalogenation of pentabromoacetone. To the combined sulphide solutions and water washings are added 1 ml. glacial acetic acid for every 5 ml. Na_2S used and a few grains of alundum or quartz. The solution is boiled to remove H_2S and to reduce the volume to about 7 ml. After cooling, 1·5 ml. $KMnO_4$ (4·7 %) are added; after about half a minute (during which the purple colour of the solution must be maintained, if necessary by further addition of $KMnO_4$) enough H_2O_2 (about 0·5 ml. '100 vol.') is added to decolorize the solution.

Titration. The solution is acidified with 2 ml. conc. HNO_3, and 1 ml. iron alum solution (100 g. $Fe(NH_4)(SO_4)_2.12H_2O$ dissolved in 100 ml. water) is added as indicator. When less than 2 mg. citric acid are expected to be present, 5 ml. 0·01042N-$AgNO_3$ are added and back-titrated with 0·01042N-KCNS. When more citric acid is present, larger quantities, or more concentrated solutions, of $AgNO_3$ must be used. 1 ml. 0·01042N-$AgNO_3$ is equivalent to 0·4 mg. anhydrous citric acid.

Discussion of special points

Preliminary ether extraction. Pucher, Wakeman & Vickery (1941) first dry plant material at 80°, acidify the powder with H_2SO_4 and extract the material with ether. It is unnecessary to carry out this procedure with animal tissues, since interfering substances are removed, without loss of citric acid, by deproteinization with metaphosphoric acid, boiling with acid, and treatment with bromine water. In our experience the ether extraction may as a rule be omitted also in the case of plant material.

Deproteinization. For testing the loss of citric acid in the course of deproteinization, a muscle suspension containing 20 g. minced muscle and 100 ml. phosphate buffer was heated to 58° for 1 hr., in order to inactivate aconitase. To 10 ml. muscle suspension were added 5 mg. citric acid (as citrate), a deproteinizing agent and water to make up to 50 ml. The recovery is shown in Table 2. The data show that recovery is complete when metaphosphoric acid is used.

Table 2. *Effect of various deproteinizing agents on the recovery of citric acid*

The concentrations stated refer to final concentrations. Citric acid was determined according to the revised method except that the concentration of H_2SO_4 was 6N during the oxidation of citric acid.

Deproteinizing agent (final concentration)	Recovery (%)
Trichloroacetic acid (3%)	90·1
Tungstic acid (0·03M)	85·5
Trichloroacetic acid (3%) + acetone (20%)	97·8
Metaphosphoric acid (5%)	100·7, 101·3, 98·2

Sources of error. The most frequent source of errors is the faulty manipulation of the petroleum extractions. Too much 'citric acid' is found if the water-washing of the petroleum is incomplete so that halide ions are left in the separating funnels, or if an accidental contamination with volatile halogen compounds (fuming HCl, $CHCl_3$, bromine vapour) occurs. Low values result from loss of petroleum extract during the washings. Special attention should therefore be paid to the careful handling of the extractions.

Recovery of citric acid. Tables 2 and 3 show that the recovery of varying quantities of citric acid from pure solutions and from tissue suspensions is satisfactory. The error does not exceed ±2·5%.

Table 3. *Recovery of citric acid from pure solutions*

Further data as in Table 2.

Citric acid		
Added (mg.)	Recovered (mg.)	Recovery (%)
0·25	0·244	97·6
1·00	0·986	98·6
2·00	2·00	100·0
4·00	4·06	101·5
10·00	10·20	102·0
20·00	20·45	102·25

Specificity of the citric acid determination. Whilst the method of Pucher *et al.* (1934) and the modification described in this paper possess a considerable degree of specificity, it

Table 4. *Specificity of the citric acid determination*

Unless stated otherwise the determination was carried out as described under 'Procedure'.

Substance	Tested (mg.)	'Citric acid' formed (mg.)	Modifications of the method
Group I			
Butyric acid	100	0·6	Solution not boiled before treatment with $KMnO_4$
Crotonic acid	100	7·8	
n-Valeric acid	100	0·4	
n-Caproic acid	100	0·5	
Group II			
Citraconic acid	100	0·4	
Mesaconic acid	100	0·6	
Citramalic acid	100	7·3	
Tricarballylic acid	100	1·6	
Group III			Conc. of H_2SO_4 before addition of $KMnO_4$
Aspartic acid	100	4·2	
Asparagine	100	1·7	
Tyrosine	100	3·8	
β-Hydroxybutyric acid	16·4	7·1	6N
	16·4	3·9	3N
	16·4	2·4	1·5N
	1·64	0·2	3N
	3·28	0·4	3N
	6·56	0·9	3N
	13·12	1·8	3N
Quinic acid	10	1·4	12N
	10	1·0	6N
	10	0·7	3N
	2	0·0	3N
	4	0·1	3N
	8	0·5	3N
	16	0·8	3N
	32	3·9	3N

must be borne in mind that biological material may contain substances other than citric acid which, under the conditions of the estimation, form either pentabromoacetone or other organic bromine compounds reacting like pentabromoacetone (i.e. passing into the petroleum phase and liberating Br ions with Na_2S).

None of the following substances when tested in quantities of 100 mg. yielded 'citric acid': succinic, fumaric, maleic, tartaric, malic, lactic, pyruvic, oxaloacetic, acetoacetic, α-hydroxyglutaric, α-ketoglutaric, acetic, malonic, glutaric, adipic, *iso*citric, *cis*- and *trans*-aconitic, acetone dicarboxylic, tiglic, meconic acids; glucose, glycerol, ethanol, ascorbic acid, glycine, alanine, glutamic acid, phenylalanine, coumarin and coumaric acid.

On the other hand, a number of substances shown in Table 4 simulate citric acid. With reference to the practical significance, these substances are classed in this table in three groups. Group I comprises lower fatty acids. As these are volatile with steam they can be removed, if present, by boiling the acid solution until the characteristic odour has disappeared. The lost water must be replaced from time to time when the volume has fallen below 35 ml. Group II contains compounds which have not been reported to occur in biological material. As a rule, therefore, they need not be considered. Group III includes substances which occur widely in biological material: aspartic acid, asparagine, tyrosine, β-hydroxybutyric and quinic acids. The first two compounds yield small quantities of 'citric acid' and therefore interfere only when their concentration is at least 10 times that of citric acid. Tyrosine can be removed by adjustment of pH to about 5·0, and filtration. β-Hydroxybutyric acid, as far as is known, occurs in animal material only. Since 1 mg. simulates about 0·2 mg. citric acid, the citric acid determination is unreliable when significant amounts of β-hydroxybutyric acid are present.

Modified colorimetric procedure in presence of interfering substances. To overcome this difficulty we make use of the fact that the bromine compound arising from β-hydroxybutyrate (which is not pentabromoacetone) forms on treatment with sulphide a very much weaker yellow colour than pentabromoacetone. Hence β-hydroxybutyric acid interferes relatively little when the colorimetric method of Pucher *et al.* (1936) is used (as already noted by these authors). The procedure for this method follows that described above up to the stage of the washing of the light petroleum extract with water. The organic bromide is then extracted successively with 3, 2 and 1 ml. of alkaline Na_2S solution. The extracts are drained into a 10 ml. measuring cylinder containing 2 ml. dioxan and further dioxan is added to make the volume up to 10 ml. After filtration the colour is compared with a standard prepared from 1 mg. citric acid. The specimen examined should not contain more than 1 mg. citric acid. The colorimetric method should also be used where aspartic acid and asparagine are present in larger quantities.

Quinic acid is known to be a common constituent of plants; Gorter (1909) claims to have detected chlorogenic acid—a depside yielding quinic and caffeic acids on hydrolysis—in the leaves of 98 out of 230 species tested. As quinic acid forms citric acid on treatment with oxidizing reagents (Fischer & Dangschat, 1934) it is not surprising that, when treated with $KMnO_4$ and Br, it forms pentabromoacetone (which was isolated and identified by m.p., mixed m.p. and Br content). Fortunately small amounts of quinic acid—below 5 mg.—yield only negligible quantities of pentabromoacetone. The problem arising from the presence of larger quantities of quinic acid and its precursors has yet to be solved.

It will be noted that both β-hydroxybutyric and quinic acids yield more 'citric acid' the stronger the acidity during the bromination. For this reason a lower degree of acidity was adopted for the routine procedure although the yield of pentabromoacetone from citric acid under these conditions is only 97%. This deficit was corrected by multiplying the result by the factor 1·031.

It follows from Table 4 that caution is needed in interpreting the results of the pentabromoacetone methods. Small quantities of 'citric acid' can be simulated by other substances. However, for the purposes of this work, i.e. for the determinations of *iso*citric and *cis*-aconitic acids, the lack of complete specificity is, as a rule, unimportant as the method essentially depends on the additional citric acid arising on incubation with aconitase.

II. ACONITASE

Measurement of aconitase activity. The activity of various tissue preparations was ascertained by polarimetric determination of the *iso*citric acid formed from *cis*-aconitic acid: 2 ml. freshly prepared 0·5 M-solution of sodium *cis*-aconitate (156 mg. *cis*-aconitic anhydride dissolved with ice-cooling in 1·5 ml. cooled 2 N-NaOH and made up to 2 ml. with water), 0·5 ml. tissue suspension (1 part tissue suspended in 5 parts medium, preserved with octanol), 2·5 ml. 0·1 M-phosphate buffer pH 7·4, and 5 ml. of water were incubated at 40° for 30 min. To stop the reaction 1·25 ml. glacial acetic acid were added. 9 ml. of this mixture, cooled in ice (to retard the formation of coloured phosphomolybdate complexes interfering with polarimetric readings), were mixed with 2 ml. M-Na_3-citrate (see Krebs & Eggleston, 1943*a*) and 9 ml. cooled 30% NH_4-molybdate. The mixture was filtered after addition of about 0·5 g. charcoal. A freshly prepared suspension of pigeon muscle gave the following readings (2 dm. tube):

min.	$\alpha_D^{20°}$
10	−0·68
20	−1·30
30	−1·51
60	−2·32
120	−2·80

Under the conditions of the measurements a reading of one degree indicated the presence of about 16 mg. *iso*citric acid in the 10 ml. of the incubated mixture.

In most of the following experiments (which were carried out under the same conditions) only one reading, after 30 min. incubation, was taken to assess the relative activity of the enzyme. It should be pointed out that the above test conditions are not suitable for the study of the equilibrium between citric and *cis*-aconitic acids because of the instability of the enzyme.

Instability of aconitase in aqueous solution. In aqueous media aconitase proved to be remarkably unstable, as shown in Table 5. Aqueous solutions are therefore unsuitable for the prolonged storage of the enzyme. It is noteworthy that fumarase is much more stable under the same conditions. As shown in Table 6, the aconitase activity of an aqueous extract had fallen to almost nil after 5 days whilst the fumarase activity was unchanged. This

confirms Jacobsohn's conclusion that fumarase and aconitase are different chemical entities (Jacobsohn, Soares & Tapadinhas, 1940).

Table 5. *Aconitase activity of aqueous muscle suspensions*

1 part minced pigeon breast muscle + 5 parts medium; the suspensions were stored in the refrigerator.

Medium	α_D (after 30 min. incubation, degrees) 1 day storage	3 days' storage	7 days' storage
Water	−1·60	−1·30	−0·47
Water	−1·86	—	−0·01
0·1 M-phosphate buffer pH 6·8	−1·50	−0·78	−0·17
0·1 M-phosphate buffer pH 7·4	−1·49	−0·89	−0·61
Acetic acid 1%	—	—	−0·10
$NaHCO_3$ 1%	—	—	−0·43

Table 6. *Comparative stability of aconitase and fumarase in extracts from pigeon breast muscle*

The conditions of testing for fumarase activity were the same as those described for aconitase, except that fumarate was used as the substrate.

		α_D (degrees) Fumarate	*cis*-Aconitate
Fresh muscle extract	15 min.	+4·81	—
	30 min.	+7·79	−1·60
Same muscle extract stored 5 days in refrigerator	15 min.	+4·85	—
	30 min.	+7·82	−0·02

Effect of glycerol on the stability of aconitase. Treatment of freshly minced tissue with 5 vol. of ice-cold ethanol or acetone and rapid drying resulted in the loss of the major part of the original aconitase activity as did drying of muscle *in vacuo* over P_2O_5.

Addition of glycerol had a stabilizing effect on aconitase in tissue extracts, as shown in Table 7. However, the relatively large quantity of glycerol necessary for the stabilization was found to interfere with the citric acid determination, by reducing very large quantities of $KMnO_4$, and we therefore abandoned the use of glycerol extracts.

Table 7. *Stabilizing effect of glycerol on aconitase*

Medium	α_D after 30 min. (degrees) 2 days' storage	5 days' storage	15 days' storage	36 days' storage
Water	−1·86	−0·20	0	0
100% glycerol	—	−1·49	−0·73	−0·69
50% glycerol (aqueous)	−1·91	−1·60	−0·23	−0·06

Stability of aconitase in frozen tissue. Freezing minced pigeon breast muscle in the freezing chamber of a refrigerator, as recommended by Cohen (1940) for the preservation of succinic dehydrogenase, stabilizes aconitase sufficiently to allow the use of the stored material over a period of several weeks. Under the test conditions the following activities were measured:

After 3 days' storage: $\alpha_D^{20°}$ −1·55°.
After 6 days' storage: $\alpha_D^{20°}$ −0·87°.
After 25 days' storage: $\alpha_D^{20°}$ −0·64°.

The procedure for the determination of *iso*citric acid, as described later in this paper, requires an activity equivalent to a rotation of at least −0·50° under the above conditions; the preservation of the enzymes by freezing is therefore satisfactory. Pigeon breast muscle can be replaced by mammalian heart muscle whose aconitase activity is only slightly lower than that of pigeon breast muscle.

Inhibition of aconitase. The effects on aconitase of a number of substances which might occur in material to be tested for *iso*citric acid were examined under standard conditions. As shown in Table 8,

Table 8. *Effect of various substances on the activity of aconitase.*

Standard conditions, frozen minced pigeon breast muscle.

Substance added	α_D (degrees) Control	With added substance
$CaCl_2$ (0·01 M)	−1·52	−1·57
$BaCl_2$ (0·01 M)	−1·52	−1·57
$MgCl_2$ (0·01 M)	−1·52	−1·63
Na-oxalate (0·1 M)	−1·16	−1·48
$CuSO_4$ (10^{-4} M)	−1·06	0
$CuSO_4$ (10^{-5} M)	−1·06	−0·74
HCN (2×10^{-3} M)	−0·89	−0·06
HCN (2×10^{-4} M)	−1·57	−1·29
Na_2S ($1·2 \times 10^{-3}$ M)	−0·89	−0·22
NaCl 2%	−1·47	−0·51
NaCl 4%	−1·47	−0·23
NaCl 8%	−1·47	−0·23
$HgCl_2$ (5×10^{-4} M)	−1·89	−0·02
$HgCl_2$ (5×10^{-5} M)	−1·89	−1·17
NaF (2×10^{-2} M)	−1·54	−1·46
Na-benzoate (4×10^{-2} M)	−1·17	−1·13
Na-iodoacetate (10^{-3} M)	−1·54	−1·36
Na-pyrophosphate ($7·5 \times 10^{-3}$ M)	−1·54	−0·60
Na-cacodylate (10^{-2} M)	−1·72	−1·50
Alloxan (10^{-2} M)	−2·32	−0·50

the chlorides of Ca, Ba and Mg (0·01 M) had no effect. Oxalate somewhat accelerated the reaction, an effect which is so far unexplained. Cu ions, $HgCl_2$, HCN and Na_2S in relatively low concentrations inhibit the enzyme. No significant inhibition was found with various animal and plant tissue extracts, but it is evidently necessary to examine the activity of aconitase in media containing untested substances before the enzyme is used.

III. PROCEDURE FOR THE DETERMINATION OF THE SUM OF (−)*iso*CITRIC AND *cis*-ACONITIC ACIDS

The material containing suitable quantities (0·5–20 mg.) of the acids to be determined should be neutral and must not contain substances which precipitate protein or otherwise inhibit aconitase.

30 ml. of the neutral solution or suspension, or smaller quantities diluted to 30 ml., are mixed in a 50 ml. graduated stoppered measuring cylinder with 5 ml. phosphate buffer (pH 7·4; 0·1M) and 5 ml. 'aconitase' (1 g. frozen pigeon breast muscle or mammalian heart muscle suspended in 5 ml. water). The mixture is placed in a water-bath at 40° for 60 min. and occasionally shaken. In a second 30 ml. sample of the material aconitase is replaced by 5 ml. water; it is otherwise treated in the same way. At the end of the incubation 20% metaphosphoric acid is added to make the volume up to 50 ml. The precipitate is filtered off into a graduated cylinder, the volume of the filtrate is measured and citric acid is determined as described in § I.

If 30 ml. of the solution or suspension do not contain enough of the acids to be determined, larger quantities are taken and the amounts of buffer, aconitase and metaphosphoric acid are increased in proportion.

Lacto-*iso*citric acid which may be present in dried material derived from certain plants does not react with aconitase (see later). If the lactone is to be included in the determination, it must be hydrolyzed before treatment with aconitase as described in § V.

Calculation. Let a = the initial amount of citric acid in the sample (as determined in the control sample not treated with aconitase); b = the amount of citric acid in the sample after incubation with aconitase; x = the sum of (−)*iso*citric and *cis*-aconitic acids in the sample. The equilibrium mixture of the three tricarboxylic acids contains (approximately) 89·5% citric acid, 6·2% *iso*citric acid and 4·3% aconitic acid (Krebs & Eggleston, 1943*a**). The concentration of *iso*citric acid is thus 6·93% and that of *cis*-aconitic acid 4·81% of the concentration of citric acid when the equilibrium is established. The final concentration of *iso*citric acid in the sample, after treatment with aconitase, is therefore $0{\cdot}0693b$, that of *cis*-aconitic acid $0{\cdot}0481b$. The sum of the initial amounts of the three acids equals the sum of the final amounts:

$$a + x = b + 0{\cdot}0693b + 0{\cdot}0481b,$$

or

$$x = 1{\cdot}117b - a. \qquad (1)$$

a, b and x in (1) refer to molecular equivalents, but not to weights. If a and b are expressed in mg., x is the sum of (−)*iso*citric and *cis*-aconitic acids expressed in mg. of *iso*citric (or citric) acid.

Accuracy of the determination of the sum of (−)*iso-citric and* cis*-aconitic acids.* The accuracy of the method largely depends on the accuracy with which the difference between a and b can be measured. The larger the amounts of citric acid relative to the amounts of *iso*citric and *cis*-aconitic acids in the sample, the greater are the errors in the determination of the difference. If the concentration of citric acid is 10 times above that of the other two acids the method becomes unreliable. If, on the other hand, the other acids prevail, their determination may be as accurate as the citric acid determination, viz. ±3%.

The accuracy would also be affected by errors in the figures for the composition of the equilibrium mixture of the three acids in the presence of aconitase. The data so far available (see Krebs & Eggleston, 1943*a*; Martius & Leonhardt, 1943) are only approximate, and the error in figure for *iso*citric acid may even be of the order of 15%; for the published values given for the specific rotation of the molybdate complex of *iso*citric acid—on which calculations of the *iso*citrate concentration have been based—differ considerably; Martius (1938) found −413°, Pucher (1942) −363°. The error in the figure for *cis*-aconitic acid is probably no more than 5%. However, the calculation according to the formula (1) does not directly depend on the accuracy of these data, but on the accuracy of the figure for the concentration of citric acid in the equilibrium mixture. Errors of 15% for *iso*citric acid and 5% for *cis*-aconitic acid, even if they were additive, would cause an error of only 1·1% in the value of citric acid (on the highly probable assumption that the equilibrium mixture contains no other acids). The factor 1·117 used in formula (1) may therefore be taken to be accurate within 1·1%.

Recovery of pure iso*citric and* cis*-aconitic acids*

*Preparation of synthetic lacto-*iso*citric acid.* To test the method synthetic preparations of the two acids were used. Fittig & Miller (1889) prepared *iso*citric acid and lacto-*iso*citric acid from trichloromethylparaconic acid by converting the latter first into Ba-*iso*citrate and decomposing the latter with H_2SO_4. According to Nelson (1930) the over-all yield is 80%. We simplified the procedure by omitting the isolation of the Ba salt.

Pure trichloromethylparaconic acid (24·7 g. 0·1 g.-mol.) (m.p. 97°; Cl content: found, 43·4%; calc. 43·0%) was dissolved in 305 ml. 2N-NaOH (A.R.) (slight excess over 0·6 g.-mol.). The solution was kept for 20 min. at 60°. Under these conditions the reaction

$$C_6H_5O_4Cl_3 + 6NaOH = Na_3C_6H_5O_7 + 3NaCl + 3H_2O$$

proceeds quantitatively as judged by the appearance of chloride ions and the disappearance of alkali. Then 32 ml. 10N-HCl (A.R.) were added (slight excess over 0·3 g.-mol.) and the solution was evaporated on a steam-bath until it was almost dry. The dish was then completely dried in a vacuum desiccator over P_2O_5 and NaOH. This required 1–2 days, and the material usually became crystalline in the desiccator. The perfectly dry mixture of crystals of lacto-*iso*citric acid and NaCl was treated with several 50 ml. portions of hot ethylacetate to dissolve the lacto-*iso*citric acid. The combined extracts were filtered and evaporated to about 80 ml. Crystals of lacto-*iso*citric acid readily

* Martius & Leonhardt (1943) found independently and with partly different methods 89·2% citric acid; 7·7% *iso*citric acid; 3·1% *cis*-aconitic acid.

appeared on cooling and the crop was much increased by gradual addition of chloroform; it was recrystallized from ethylacetate, m.p. 163°. Yield 11·5 g. (60% of calc.).

Preparation of cis-*aconitic acid.* *cis*-Aconitic anhydride, m.p. 78°, prepared according to Malachowski & Maslowski (1928), contained an impurity even after repeated recrystallization from benzene, as indicated by the yellow colour arising on addition of alkali to the aqueous solution and by incomplete yields of citric acid on treatment with aconitase (Table 9). A pure sample of the acid was prepared as follows: 3·12 g. (0·02 g.-mol.) *cis*-aconitic anhydride (m.p. 77°) were dissolved in about 25 ml. warm ethyl acetate which had been saturated with water at room temperature. Chloroform was added until the solution became turbid. The acid crystallized in white needles, m.p. 130° (5° higher than reported by Malachowski & Maslowski, 1928). Analysis (Drs Weiler and Strauss, Oxford): found: C, 40·97; H, 3·22%. Calc. C, 41·38; H, 3·47%. Alkaline solutions of the acid are colourless. The crystals are stable for at least 3 months at room temperature with exposure to air.

Recovery. Table 9 shows that recovery from pure solution is satisfactory, the error of a single analysis being no more than 4%.

Table 9. *Recovery of* iso*citric acid and* cis-*aconitic acid from pure solutions*

Substrate added (mg.)	*iso*Citric or *cis*-aconitic acids: Expected* (mg.)	Found† (mg.)	Recovery (%)
*r-iso*Citric acid lactone‡ 10·9	6·08	6·26	103
11·35	6·35	6·28	99
19·2	9·60	9·76	102
cis-Aconitic anhydride§ 7·80	8·70	8·30	95
7·80	8·70	8·03	92
3·90	4·35	4·16	96
3·90	4·35	4·02	92
cis-Aconitic acid 9·45	9·45	9·28	98
8·7	8·7	8·63	99
8·7	8·7	8·35	96
8·7	8·7	8·50	98

* In the case of synthetic *iso*citric acid the expected yield is 50% of the total acid (Martius, 1938).

† A correction was made allowing for a yield of 97% of citric acid.

‡ Lacto-*iso*citric acid was hydrolyzed for 20 min. at 40° with 0·01 N-NaOH.

§ Probably impure; see text.

IV. SOME REACTIONS OF *cis*-ACONITIC AND *iso*CITRIC ACIDS

As a preliminary to work on the separate determination of the two acids it was necessary to study: (*a*) the stability of *cis*-aconitic acid and the interconversion of the *cis* and *trans* acids; (*b*) the interconversion of *iso*citric and lacto-*iso*citric acids; (*c*) the behaviour of lacto-*iso*citric acid towards enzymes.

Known quantities of the acids were exposed to varying conditions and their concentration was measured by the method described in the preceding section. The method permits the determination of *cis*-aconitic acid in the presence of *trans*-aconitic acid, and of *iso*citric acid in the presence of its lactone, for *trans*-aconitic acid and lacto-*iso*citric acid do not react in the presence of aconitase to form citric acid.

Stability of cis-*aconitic acid in aqueous solutions.* Few details are known about the stability of *cis*-aconitic acid and its salts. Malachowski & Maslowski (1928) state that in aqueous solution *cis*-aconitic acid is completely converted into the *trans* form in 5 min. at 100° and that at 60° the major part of the acid is transformed in 10 min. Our data (Table 10) roughly confirm these statements, but they show that the neutral solutions of the Na salt are relatively stable. After 24 hr. at 20° or 39° less than 4% disappeared. In 5 days at 39° about 10% disappeared. Even at 100° the change is slow: after 2 hr. 12% were lost. In acid or alkaline solutions *cis*-aconitic acid is less stable. In 0·1 N-HCl about 90% and in 0·1 N-NaOH about 80% disappeared at 100° within 1 hr. Under none of the test conditions, it will be noted, was the conversion into the *trans* form complete.

Table 10. *Stability of* cis-*aconitic acid and its salts in aqueous solutions*

Medium containing 8·7 mg. *cis*-aconitic acid	Temp.	Time	*cis*-Aconitic acid found (mg.)	*cis*-Aconitic acid removed, as % of added acid
5 ml. water	100°	10 min.	7·66	11·9
1 ml. water	40°	3 days	1·14	86·7
5 ml. 0·1 N-HCl	100°	1 hr.	0·92	89·3
,,	100°	2 hr.	0·79	90·8
5 ml. 0·5 N-HCl	100°	10 min.	2·16	74·9
10 ml. N-HCl	40°	2 days	1·34	84·5
5 ml. water; neutralized with Na_2CO_3	40°	24 hr.	8·08	5·9
,,	40°	5 days	7·53	13·4
,,	100°	2 hr.	7·27	16·4
10 ml. M-Na_2CO_3	40°	2 days	6·80	20·9
5 ml. 0·5 M-Na_2CO_3	100°	10 min.	6·85	20·2
5 ml. 0·1 N-NaOH	100°	1 hr.	1·83	78·7
5 ml. N-NaOH	100°	10 min.	1·25	85·5

Conversion of trans-*aconitic acid into* cis-*aconitic acid.* A specimen of 'aconitic acid' obtained from British Drug Houses Ltd., m.p. 191°, was used. It contained less than 0·1% citric acid. A freshly prepared aqueous solution was free from *cis*-aconitic acid. Heating aqueous solutions of the acid and its salts to 100° produced considerable quantities of *cis*-aconitic acid, as shown in Table 11, the yields exceeding under some conditions 30%. The extent of the conversion depends much on the H^+ concentration. Virtually no *cis*-aconitic acid is formed in neutral solutions, but the conversion takes place on both sides of the neutral point.

When aqueous solutions of *trans*-aconitic acid are dried at 100° and kept at this temperature for some time much *cis*-aconitic acid is formed under certain conditions as shown

Table 11. *Conversion of* trans-*aconitic acid into* cis-*aconitic acid in aqueous solutions*

trans-Aconitic acid added (mg.)	Medium	Temp.	Time (min.)	*cis*-Aconitic acid formed* (mg.)	*cis*-Aconitic acid formed, as % of added acid
69·6	5 ml. water	100°	30	8·46	12·1
69·6	5 ml. water; neutralized with $NaHCO_3$	100°	30	0·14	0·2
348	5 ml. 0·1 N-HCl	100°	30	39·3	11·3
348	,,	100°	60	38·8	11·1
34·8	,,	100°	60	11·8	33·9
17·4	,,	100°	60	2·88	16·5
8·7	,,	100°	60	1·89	21·7
348	5 ml. N-HCl	100°	30	39·5	11·5
69·6	0·8 ml. N-$NaHCO_3$ + 2 ml. water	100°	300	16·3	23·4
69·6	0·4 ml. N-$NaHCO_3$ + 2 ml. water	100°	300	20·5	29·5
69·6	5 ml. N-NaOH	100°	30	7·33	10·8
69·6	0·8 ml. N-$NaHCO_3$ + 2 ml. water	140°†	120	10·4	14·9
69·6	0·4 ml. N-$NaHCO_3$ + 2 ml. water	140°†	120	2·82	4·1

* Controls showed that no citric acid was formed.

† Heated in autoclave, pressure 40 lb./sq. in.

in Table 12. The neutral salt and the free acid formed no *cis*-isomeride, the monosodium salt 31·6%, the disodium salt 12·1%. No *cis*-aconitic acid was formed when the solution of the Na and Na_2 salts were dried *in vacuo* at room temperature. These observations are relevant when plant material is prepared for analysis (§ VI).

Table 12. *Conversion of* trans-*aconitic acid into* cis-*aconitic acid on evaporation of aqueous solutions*

Treatment of 69·6 mg. *trans*-aconitic acid dissolved in 2 ml. water	*cis*-Aconitic acid formed (mg.)	*cis*-Aconitic acid formed, as % of added acid
Dried at 100°; kept dry at 100°, 2 hr.	0·2	0·3
+0·4 ml. M-$NaHCO_3$ (to form mono-Na salt); dried at 100°; kept dry at 100°, 2 hr.	22·0	31·6
+0·8 ml. M-$NaHCO_3$ (to form di-Na salt); dried at 100°; kept dry at 100°, 2 hr.	8·45	12·1
+0·4 ml. M-$NaHCO_3$ (mono-Na salt); dried in vacuum desiccator, 24 hr., room temp.	0·1	0·14
+0·8 ml. M-$NaHCO_3$ (di-Na salt); dried in vacuum desiccator, 24 hr., room temp.	0	0
+1·2 ml. M-$NaHCO_3$ (to form tri-Na salt); kept dry at 100°, 2 hr.	0·1	0·14

The ready conversion of the *trans* into the *cis* form may be responsible for the fact that the m.p. of pure *trans*-aconitic acid shows considerable variations. Malachowski & Maslowski (1928) remark that specimens melting at 182·5 and 194·5° both gave correct analytical values.

Hydrolysis of iso*citric acid lactone.* When 0·2 ml. *iso*citric acid lactone was added to 3 ml. 0·02 M-$NaHCO_3$, saturated with 5% CO_2 in O_2, 127 μl. CO_2 were liberated (measured manometrically). The evolution of CO_2 was completed after 5 min. at 40°. During the following 60 min. no measurable quantities of CO_2 were evolved. On the assumption that the lactone is not hydrolyzed, 125 μl. CO_2 were calculated. Thus at 40° and pH 7·3 the rate of hydrolysis of the lactone is insignificant.

The rate of hydrolysis by alkali under other conditions was measured in the following way: a known weight of lactone (about 20 mg.) was dissolved in 0·01 N-$NaHCO_3$ or NaOH. The solutions were kept for varying periods at different temperatures and at the end of the period 3 ml. were pipetted into a conical Warburg vessel provided with a side-arm containing 0·3 ml. 10% H_2SO_4. The gas space of the vessel was filled with a gas mixture containing 5% CO_2. The CO_2 combined with the excess of alkali in the solution and the bicarbonate content of the solution was measured manometrically in the usual way. The difference in the amount of bicarbonate between the control containing no *iso*citric acid and the *iso*citrate solution indicates the amounts of acidic groups. The method permits the measurement of the degree of the hydrolysis of the lactone on 1–2 mg. material with an accuracy of about 2%.

In bicarbonate solution (pH between 7 and 8) 18% of the lactone was hydrolyzed in 30 min. at 100°. In dilute NaOH (initial concentration 0·01 N and the excess NaOH after complete hydrolysis being about 0·003 N) the hydrolysis was complete within 10 min. at 100°. At 40° the following rate was observed, when 34·8 mg. lacto-*iso*citric acid were dissolved in 20 ml. 0·04 N-NaOH (required for neutralization of *iso*citric acid, 20 ml. 0·03 N; thus excess of alkali after neutralization was 0·01 N). After 10 min. 91·2%, after 30 min. 98·5%, after 90 min. 99·2% were split. To effect hydrolysis it is therefore sufficient to add an excess of 0·01 N-NaOH and to keep the solution for 30 min. at 40°.

The lactone is also readily hydrolyzed, though not to completion, on heating in acid solution; 12·65 mg. synthetic lactone were dissolved in 10 ml. 0·5 N-HCl and heated for 30 min. at 100°. After cooling, the solution was neutralized with M-$NaHCO_3$. The *iso*citric acid determination yielded 5·36 mg. (calc. for complete hydrolysis, 6·37 mg.).

*Behaviour of lacto-*iso*citric acid towards enzymes.* As already reported by Martius (1938) and confirmed by Table 8, 50% of synthetic *iso*citric acid reacts with aconitase. Lacto-*iso*citrate forms no citric acid when incubated with muscle or liver suspensions at pH 7·4. These tissues thus do not contain enzymes capable of hydrolyzing the lactone.

V. SEPARATE DETERMINATION OF (−)*iso*-CITRIC ACID AND *cis*-ACONITIC ACID

The separate determination of these two acids met with unexpected difficulties when the following principle was applied. In a measured portion of the material the sum of the acids was measured. A second portion was acidified and evaporated to dryness. As shown in Table 13, *cis*-aconitic acid is quantitatively removed from pure solutions by this treatment and it was expected that thus the remaining *iso*citric acid could be determined separately; the difference between the results of the first and second analyses would then be the amount of *cis*-aconitic acid in the sample. However, whilst it is possible to remove *cis*-aconitic acid from pure solutions, we find that its conversion into the *trans* form is not quantitative when certain other substances are present; among these are citric acid, *iso*citric acid and plant tissues. Under the conditions tested, between 4 and 16% of the added *cis*-aconitic acid remained unchanged. We have not yet found a method which removes *cis*-aconitic acid quantitatively without affecting the concentration of *iso*citric acid, and as yet the following procedure is the best available for the separate determination of the two acids. The method gives correct values for the sum of the acids, or for the individual acids when present alone. When both are present in about equal quantities the values for *iso*citric acid are between 10 and 20% too high, those for *cis*-aconitic between 10 and 20% too low.

Procedure. In the first two measured portions the sum of the two acids is analyzed as already described. A further portion, in a porcelain evaporating dish (about 8 cm. diameter), is acidified with 1 ml. conc. HCl. The dish is heated on a steam-bath for 30 min. after the evaporation of the water. After cooling, the material is dissolved or suspended in 10–15 ml. water, quantitatively transferred to a 50 ml. stoppered measuring cylinder and made alkaline to phenol red with 2N-NaOH. A further excess of 1 ml. 2N-NaOH is added to hydrolyze the *iso*citric lactone. Incubation at 40° completes the hydrolysis within 30 min. The next step is the adjustment of the pH to 7·2–7·4 (orange to phenol red) with M-H_3PO_4. The material is diluted to 35 ml. and is then ready, without further addition of buffer, for treatment with aconitase as described in § III.

Calculation. Let a = the initial amount of citric acid; b = the amount of citric acid after treatment with aconitase in the sample containing *cis*-aconitic acid; b' = the final amount of citric acid after treatment with aconitase in the sample from which *cis*-aconitic acid has been removed; y = the amount of (−)*iso*citric acid in the sample; z = the amount of *cis*-aconitic acid in the sample. Then

$$y+z = 1{\cdot}117b - a \text{ (as previously shown);}$$
$$y = 1{\cdot}117b' - a;$$
$$z = 1{\cdot}117\,(b - b').$$

If a, b and b' are expressed in mg. (instead of mM.) the last equation becomes

$$z = 1{\cdot}102\,(b - b').$$

Recovery. Applying the foregoing procedure to pure mixtures of (−)*iso*citric and *cis*-aconitic acids, we found the following recovery:

Added (mg.)			Recovered (mg.)		
(−)*iso*-Citric acid*	*cis*-Aconitic acid	Sum	Sum	*iso*-Citric acid	*cis*-Aconitic acid†
5·39	4·01	9·40	9·48	6·05	3·43
4·80	5·72	10·52	10·52	5·58	4·94

* Added as *r*-*iso*citric acid.
† Expressed in mg. (equimolar) citric acid.

Whilst recovery of the sum of the two acids was satisfactory the yield of *iso*citric acid was too high and that of *cis*-aconitic acid too low. In other words, part of the *cis*-aconitic acid appeared as *iso*citric acid. This was not due to a formation of *iso*citric (or citric) acid from aconitic acid during the treatment with hot HCl and dry heat, but to the incomplete conversion of the *cis* into the *trans* form.

Further data showing the effect of various substances on the conversion of *cis*- into *trans*-aconitic acid are recorded in Table 13. Only when no other substances are present is *cis*-aconitic acid quantitatively removed on evaporation of the acid solution.

Table 13. *Removal of* cis*-aconitic acid in the presence of other substances by treatment with* HCl *and evaporation to dryness*

For details of procedure see text

cis-Aconitic acid added (expressed in mg. of equimol. citric acid)	Other substances added	'*iso*Citric acid' found (mg.)	*cis*-Aconitic acid not converted into *trans* form (as mg. citric acid)	*cis*-Aconitic acid appearing as '*iso*citric acid' (as % of added *cis*-aconitic acid)
6·25	None	0	0	0
5·60	None	0	0	0
6·25	5·50 mg. (−)*iso*citric acid	6·01	0·51	8·1
6·35 *	6·10 mg. citric acid	0·37	0·37	5·8
6·35 *	6·10 mg. citric acid	0·33	0·33	5·2
6·33	5·55 mg. (−)*iso*citric acid	6·30	0·75	11·9
6·33	5·85 mg. (−)*iso*citric acid	6·42	0·57	9·0
12·66	5·85 mg. (−)*iso*citric acid	6·48	0·63	3·5
6·57	400 mg. dried foxglove leaves containing 3·50 mg. citric acid and 7·57 mg. *iso*citric acid	8·83	0·96	14·6
6·30	400 mg. dried leaves of *Delphinium* containing 2·35 mg. citric acid	1·02	1·02	16·2

* Duplicates.

As yet the available evidence does not indicate that *cis*-aconitic acid occurs in biological material except in very small quantities as an accompaniment of citric acid in animal tissues (and under artificial experimental conditions). In many cases, therefore, *iso*citric acid will be present alone, so that no error will result from incomplete removal of *cis*-aconitic acid.

Specificity of the method. The specificity of the determination of *iso*citric and *cis*-aconitic acids depends on the specificity of the aconitase preparation used. The two tricarboxylic acids are the only substances at present known to form citric acid under the conditions of the test. Although fresh muscle can under certain conditions form citrate when oxaloacetate is available, this does not apply to dilute suspensions of frozen muscle as used in the above procedure. There is therefore good reason to assume that the specificity of the method is very high, if not absolute. Thus if incubation with aconitase increases the amounts of citrate this increase is in all probability due to *iso*citrate or *cis*-aconitate. If, however, citric acid is present in the material, an error can arise from the fact that the specificity of the determination of citric acid is not absolute. The calculation of the amounts of *iso*citric and *cis*-aconitic acid is based on the assumption that addition of aconitase removes 10·5% of the preformed citric acid. Any 'pseudo' citric acid would not be attacked by aconitase and therefore simulates *iso*citric or *cis*-aconitic acid in an amount equivalent to 10·5% of the 'pseudo' citric acid.

It follows that if the citric acid determination shows no increase after incubation or with aconitase such values of '*iso*citric' or '*cis*-aconitic' acids as may be arrived at by applying formula (1) are somewhat uncertain.

VI. PREPARATION OF BIOLOGICAL MATERIAL

Extracts from animal tissues should be neutralized and, as already pointed out, must not contain substances which inhibit aconitase. Animal tissues should be minced or otherwise broken up, but it is not necessary to remove the tissue particles or proteins before the addition of aconitase. The methods as described can therefore be applied to suspensions of animal tissues without modification.

Since plants may contain *trans*-aconitic acid—it has been isolated from many species*—special care is required to avoid the artificial formation of *cis*-aconitic acid from the *trans* form. Drying of plant material at high temperatures, as practised for general analytical purposes by Pucher *et al.* (1941), is evidently unsatisfactory (see Tables 11 and 12). On the other hand, drying and pulverizing plant tissue is advantageous because it disrupts the tissues and facilitates the extraction of the cell contents. We dry the material—5–10 g. wet weight—in a vacuum desiccator over P_2O_5; the dried tissue is either ground in a mortar, or, in the case of fibrous material, chopped up with scissors. Portions of suitable size varying between 50 and 500 mg. according to the tricarboxylic acid content are used for the analysis. They are suspended in water and then treated in the same way as suspensions of animal tissues.

An ether extract prepared according to Pucher *et al.* (1941) may be used instead of a suspension of dried material.

VII. SOME APPLICATIONS OF THE METHOD

iso*Citric acid in plants.* Application of the method confirms Pucher & Vickery's (1942) discovery that the so-called 'Crassulacean malic acid' is (−)*iso*citric acid. Table 14 shows that the leaves of the seven species examined contained between 3·8 and 12·7% *iso*citric acid (calculated on a dry-weight basis). *cis*-Aconitic acid was not present in measurable quantities.

No appreciable amount of *iso*citric or *cis*-aconitic acid was found in the leaves of the following species: *Epilobium angustifolium* L., *Saxifraga umbrosa* L., *Cucumis sativa* L., *Geranium pratense* L., *Geranium*

* It is not impossible that *trans*-aconitic acid isolated from plants arises from the *cis*-acid during the process of isolation. This question is under investigation.

Table 14. iso*Citric acid in the leaves of Crassulaceae*

The leaves were collected between 6 and 7 a.m. during 19 June and 3 July 1943 with the exception of those of *Sedum praealtum* which were collected on 5 May 1944.

Species	Dry leaf analyzed (mg.)	Citric acid found (mg.)	*iso*Citric acid found (mg.)	Percentage content (calculated for dry wt.)	
				Citric acid	*iso*Citric acid
Bryophyllum calycinum Salisb.	50	1·94	5·46	3·88	10·92
Sempervivum tectorum L.	200	4·60	14·20	2·30	7·10
Sempervivum montanum L.	100	2·29	12·73	2·29	12·73
Sedum album L.	200	2·76	14·80	13·8	7·40
Sedum acre L.	80	1·27	3·01	1·57	3·76
Sedum praealtum Sol.	100	2·29	10·80	2·29	10·80
Escheveria sp.	50	0·465	2·42	0·93	4·84

sanguineum L., *Pelargonium* sp., *Solanum tuberosum* L. ('Duke of York'), *Rubus fruticosus* L., *Helianthus annuus* L., *Lysimachia nummularia* L., *Vicia faba* L. (broad bean).

Considerable quantities of *iso*citric acid (1–3 % of the dry weight) were found in the leaves of the foxglove (*Digitalis purpurea* L.). A sample of leaves collected from first-year plants in June gave the following results:

Before treatment with aconitase:	3·18 mg.	Citric acid in 400 mg. dry leaves
After treatment with aconitase:	9·88 mg.	
After removal of *cis*-aconitic acid:		
Before treatment with aconitase:	3·20 mg.	
After treatment with aconitase:	9·71 mg.	
Thus Citric acid content:	0·80 %	
*iso*Citric acid content:	1·93 %	
cis-Aconitic acid content:	Negligible	

Diurnal changes in Bryophyllum calycinum *and* Sedum praealtum. It has long been known that the concentration of titratable acid of the leaves of *Bryophyllum* and other Crassulaceae increases during darkness and this has been attributed mainly to changes in the malic acid concentration (see Bennet-Clark, 1933). Since the term 'malic acid' in this context was usually meant to include 'Crassulacean malic acid' it seemed of interest to re-examine the nature of the acid changes.

Tables 15 and 16 confirm (see Bennet-Clark, 1933) that the diurnal changes chiefly, though not exclusively, affect malic acid. In *Bryophyllum* 83 % of the changes in concentration of organic acid is due to malic acid, 8 % to *iso*citric acid and 9 % to citric acid; in *Sedum* 79 % is due to malic acid, 9 % to *iso*citric acid and 12 % to citric acid. It will be noted that the changes in organic acid concentration considerably exceed the changes in titratable acidity. As already pointed out by Bennet-Clark this indicates that part of the organic acid in the leaves is ionized.

SUMMARY

1. A modification of the volumetric citric acid determination of Pucher *et al.* (1934) is described and its specificity is examined. β-Hydroxybutyric and quinic acids are the most common substances in biological material which simulate citric acid.

Table 15. *Diurnal changes in the composition of the leaves of* Bryophyllum calycinum

'Light' refers to leaves collected at 3 p.m. on a sunny day (23 June 1943); 'dark' refers to leaves collected from the same plant after it had been kept in complete darkness at 23° for 16 hr.

	100 mg. dry* leaves contain				
	Light		Dark		Changes caused by transfer to darkness
	(mg.)	(μ-equiv.)	(mg.)	(μ-equiv.)	
Total titratable acidity	—	96	—	205	+109 μ-equiv.
*iso*Citric acid	10·2	159	10·9	171	+ 12 μ-equiv.
Citric acid	3·0	48	3·9	61	+ 13 μ-equiv.
Malic acid	4·5	67	12·8	191	+124 μ-equiv.
Succinic and fumaric acids	Trace	—	Trace	—	—
Sum of *iso*citric, citric, malic acids	—	274	—	423	+149 μ-equiv.
Total carbohydrates†	36·0	—	29·8	—	− 6·2 mg.

* The dry weight was 6·98 % of the wet weight.
† Reducing sugar after hydrolysis, expressed as glucose; according to Bennet-Clark (1933) a proportion of the carbohydrate is probably sedoheptose.

Table 16. *Diurnal changes in the composition of the leaves of* Sedum praealtum

'Light' refers to leaves collected at 2.15 p.m., 5 May 1944, after direct exposure to bright sunlight throughout the day; 'dark' refers to leaves collected from the same plant after it had been kept in complete darkness for 18 hr. at 25°.

	100 mg. dry* leaves contain				
	Light		Dark		Changes caused by transfer to darkness
	(mg.)	(μ-equiv.)	(mg.)	(μ-equiv.)	
Total titratable acidity	—	22	—	162	+140 μ-equiv.
*iso*Citric acid	12·5	195	13·7	214	+ 19 μ-equiv.
Citric acid	0·9	14	2·4	38	+ 24 μ-equiv.
Malic acid	5·3	79	15·9	238	+159 μ-equiv.
Sum of *iso*citric, citric, malic acids	—	288	—	490	+202 μ-equiv.
Total carbohydrate†	18·2	—	9·6	—	− 8·6 mg.
'Glucose'‡	8·4	—	2·2	—	− 6·2 mg.

* The dry weight was 4·84 % of the wet weight. † Reducing sugar after acid hydrolysis.
‡ Reducing sugar (Hagedorn-Jensen) expressed as glucose (the sugar is largely sedoheptose (Bennet-Clark, 1933)).

When β-hydroxybutyric acid is present the colorimetric method of Pucher *et al.* (1936) is preferable; the acid does not interfere with this method as long as its concentration is not more than 10 times higher than that of citric acid.

2. For the determination of (−)*iso*citric acid and *cis*-aconitic acids the material is divided into three equal portions. The first is treated with aconitase, and the citric acid formed from *iso*citric acid and *cis*-aconitic acid under the influence of this enzyme is determined. In the second, citric acid is determined directly. From the third portion *cis*-aconitic acid is removed by conversion into the *trans* form before it is treated in the same way as the first sample. The difference in the citric acid content between the first and second portions indicates the sum of (−)*iso*citric and aconitic acids, the difference between the second and third the amount of (−)*iso*citric acid, the difference between the first and third the amount of *cis*-aconitic acid.

3. An aconitase preparation suitable for analytical work is described and its properties are examined. Unlike fumarase, aconitase is unstable in aqueous tissue extracts. The enzyme is fairly stable in frozen tissue; it is inhibited by Cu ions, $HgCl_2$, HCN and H_2S.

4. Methods for the preparation of *r*-lacto-*iso*citric and of *cis*-aconitic acids are described.

5. The stability of *cis*-aconitic acid and the interconversion of *cis*- and *trans*-aconitic acids is investigated, the measurements being based on the fact that aconitase reacts with the *cis* form only. *cis*-Aconitic acid was found to be much more stable than data in the literature suggest; e.g. in neutral solution only 4% disappeared in 24 hr. at 20°; at 100° 12% was lost in 2 hr. *trans*-Aconitic acid is converted into the *cis* form in acid or alkaline solution at 100°; the yields may exceed 30%.

6. Pucher & Vickery's (1942) discovery that the 'Crassulacean malic acid' is (−)*iso*citric acid is confirmed. Leaves of *Bryophyllum*, *Sedum*, *Sempervivum* and *Escheveria* were found to contain between 3·8 and 12·8% (−)*iso*citric acid (calculated for dry weight).

7. The diurnal changes in titratable acidity in leaves of *Bryophyllum calycinum* and *Sedum praealtum* were found to be chiefly due to changes in the malic acid concentration.

We are indebted to the Rockefeller Foundation and the Medical Research Council for grants, to the Curator of the Royal Botanical Gardens, Kew, for a specimen of *Bryophyllum calycinum*, and to Prof. Bennet-Clark for a specimen of *Sedum praealtum*.

REFERENCES

Bennet-Clark, T. A. (1933). *New Phytol.* **32**, 39, 128, 197.
Cohen, P. P. (1940). *J. biol. Chem.* **136**, 565.
Evans, E. A., Jr. & Slotin, L. (1941). *J. biol. Chem.* **141**, 439.
Fischer, H. O. L. & Dangschat, G. (1934). *Helv. chim. Acta*, **17**, 1196.
Fittig, R. & Miller, H. E. (1889). *Liebigs Ann.* **255**, 43.
Gorter, K. (1909). *Arch. Pharm., Berl.*, **247**, 184.
Jacobsohn, K. P., Soares, M. & Tapadinhas, J. (1940). *Bull. Soc. Chim. biol., Paris*, **12**, 48.
Johnson, W. A. (1939). *Biochem. J.* **33**, 1046.
Krebs, H. A. (1943). *Advances in Enzymology*, **3**, 191.
—— & Eggleston, L. V. (1943*a*). *Biochem. J.* **37**, 384.
—— —— (1943*b*). *Biochem. J.* **37**, *Proc.* xv.
—— Smyth, D. H. & Evans, E. A., Jr. (1940). *Biochem. J.* **34**, 1041.
Malachowski, R. & Maslowski, M. (1928). *Ber. dtsch. chem. Ges.* **61**, 2521.
Martius, C. (1938). *Hoppe-Seyl. Z.* **257**, 29.
—— & Leonhardt, H. (1943). *Hoppe-Seyl. Z.* **278**, 208.
Nelson, E. K. (1930). *J. Amer. chem. Soc.* **52**, 2928.
Pucher, G. W. (1942). *J. biol. Chem.* **145**, 511.
—— Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.
—— & Vickery, H. B. (1942). *J. biol. Chem.* **145**, 525.
—— —— & Leavenworth, C. S. (1934). *Industr. Engng Chem.* (Anal. ed.), **6**, 190.
—— Wakeman, A. J. & Vickery, H. B. (1941). *Industr. Engng Chem.* (Anal. ed.), **13**, 244.
Wood, H. G., Werkman, C. H., Hemingway, A. & Nier, A. D. (1941). *J. biol. Chem.* **139**, 483.

Human-milk Fat

2. COMPONENT GLYCERIDES

By T. P. HILDITCH and M. L. MEARA, *Department of Industrial Chemistry, University of Liverpool*

(*Received* 1 *September* 1944)

The component acids of human-milk fat were shown in the preceding part of this series (Hilditch & Meara, 1944) to differ in several respects from those of cow-milk fat. Although palmitic, stearic and myristic acids are present in proportions similar to those in which they occur in cow-milk fats, the lower saturated acids are quite different, comprising only lauric and small proportions of decanoic acid. Again, in the unsaturated series, although oleic acid forms 30–37% (by wt.) of the total fatty acids, it is

accompanied by 6–8 % of octadecadienoic acids, the greater part of the latter being (contrary to what is observed in cow and some other milk fats) the ordinary linoleic acid which occurs abundantly in the vegetable kingdom. The further study of the component mixed glycerides of human-milk fat which is now communicated throws some light on the distribution of these acids in the glycerides of the fat, and also provides data for comparison of the general structure of the human-milk fat glycerides with those of cow-milk fat (Hilditch & Paul, 1940).

METHODS

The method employed was to crystallize the human-milk fat (430 g.) systematically from acetone at various temperatures between 0 and −25°, when eventually five fractions were obtained (cf. Table 1). Each of these fractions was submitted to examination for component fatty acids by the ester-fractionation procedure, and the two least soluble, most saturated fractions (A and B) were also analyzed for fully saturated glycerides (by oxidizing mixed saturated-unsaturated components to acidic derivatives with potassium permanganate in acetone).

The specimen of human-milk fat was assembled from four samples of similar iodine value (i.v.), namely, 120 g. of i.v. 60·4, 108 g. of i.v. 60·3, 161 g. of i.v. 58·9 and 43 g. of i.v. 58·4. The iodine value of the mixed specimen was 60·1. The conditions of crystallization were briefly as follows:

(i) The fat (430 g.) was first dissolved in acetone (5 ml./g.) and left at 0° for 7 days, when the deposited crystals were removed by filtration, and again crystallized from acetone (5 ml./g.) at 0° for 5 days; 101·7 g. (i.v. 26·4) of a least-soluble fraction (A) was thus obtained.

(ii) The fat was recovered from both acetone filtrates, and next crystallized from acetone (5 ml./g.) at −25° for some hours, when a considerable proportion separated and was removed, leaving 156·0 g. (i.v. 95·9) of a most soluble fraction (D).

(iii) The portion (172·3 g.) separated at −25° was further systematically crystallized three times at temperatures of 0, −5 and −10°, and was ultimately resolved into two intermediate fractions: B, 80·5 g., i.v. 36·6, and C, 91·8 g., i.v. 57·4.

RESULTS

Component acids of the individual human-milk fat fractions. A portion of each fraction was hydrolyzed and the resulting acids submitted to lead salt separation from ethanol; each group of acids was converted into methyl esters, which were submitted to distillation in a vacuum through an electrically heated and packed column, the resulting methyl-ester fractions being examined for equivalent and iodine value. From the data obtained the proportions of the total component acids of each fraction were calculated, the final results only in each case being quoted in Table 1.

Inspection of Table 1 reveals that the octadecadienoic or linoleic acid is concentrated in the most soluble glycerides. This is in accordance with the general 'rule of even distribution', since oleic acid, the most abundant acid in the milk fat, is present in proportions which involve its occurrence at least once in a very large proportion of the triglyceride molecules. Concurrent presence of a linoleic group therefore necessitates that the glycerides which contain the latter also include two (or even three) unsaturated acid groups in their molecules, i.e. belong to a group which are more readily soluble in acetone than mono-unsaturated, di-saturated, or fully saturated glycerides.

The proportions of the other acids in the four fractions also show the general relationships characteristic of natural mixtures of mixed glycerides after segregation into fractions of differing solubility in acetone.

Table 1. *Human-milk fat fractions from acetone*

	A		B		C		D	
Weight (g.)	101·7		80·5		91·8		156·0	
Iodine value	26·4		36·6		57·4		95·9	
Saponification equiv.	271·7		272·6		277·0		280·9	
Glycerides: (% by wt.)	23·7		18·7		21·3		36·3	
(% by mol.)	24·0		19·0		21·3		35·7	
Component acids:	(% by wt.)	(% by mol.)	(% by wt.)	(% by mol.)	(% by wt.)	(% by mol.)	(% by wt.)	(% by mol.)
Decanoic	1·1	1·7	0·6	0·9	0·9	1·4	2·2	3·4
Lauric	5·1	6·6	6·1	7·9	5·1	6·7	6·8	9·0
Myristic	9·9	11·3	10·1	11·5	9·1	10·5	4·6	5·3
Palmitic	39·4	39·8	33·7	34·2	23·0	23·6	6·8	7·0
Stearic	15·4	14·0	10·4	9·5	5·5	5·1	0·5	0·5
Arachidic	2·4	2·0	1·1	0·9	1·2	1·0	—	—
Decenoic	—	—	—	—	Trace	Trace	0·1	0·1
Dodecenoic	0·1	0·1	0·1	0·1	0·5	0·7	0·2	0·3
Tetradecenoic	0·1	0·1	0·7	0·8	0·6	0·7	1·1	1·3
Hexadecenoic	4·0	4·0	2·6	2·6	4·3	4·4	8·0	8·3
Oleic	17·8	16·4	28·7	26·4	39·0	36·2	49·5	46·4
Octadecadienoic	1·1	1·1	2·9	2·7	7·5	7·0	14·7	13·8
Unsaturated C_{20-22}	3·6	2·9	3·0	2·5	3·3	2·7	5·5	4·6

Fully saturated glycerides in fractions A and B. Fraction A. A portion (50·6 g.) was oxidized with potassium permanganate in acetone and yielded 17·5 g. fully saturated glycerides which, on crystallization from acetone (175 ml.) at room temperature, gave (*a*) 13·22 g., the component acids of which were decanoic 1·5, lauric 13·3, myristic 22·8, palmitic 38·0, stearic 20·8, and arachidic 3·6 % (by mol.); and (*b*) 3·84 g., i.v. 3·0, acid value 21·1, corresponding to 2·52 g. (corrected) fully saturated glycerides. The content of fully saturated glycerides in fraction A was therefore 31·1 % (by wt.) or 32·4 % (by mol.).

Fraction B. A portion (38·6 g.) left after oxidation 3·85 g. crude fully saturated glycerides (i.v. 2·3, acid value 21·5), corresponding to 2·59 g. fully saturated glycerides (saponification equiv. 252·0), i.e. 6·7 % (by wt.) or 7·2 % (by mol.) of fraction B. This small amount did not permit an ester-fractionation analysis of the component acids, which have been taken for purposes of calculation as equal proportions of (i) palmitic and (ii) myristic and lauric acids.

The total amount of fully saturated glycerides in the human-milk fat was thus 9·1 % (by mol.), whilst its component acids (cf. Table 2) were made up of 49·8 % saturated and 50·2 % unsaturated acids. A fat of this composition which follows the rule almost invariably operative in seed fats and many other fats would contain negligible proportions of fully saturated glycerides; on the other hand, the observed proportion falls on the curve connecting the fully saturated glyceride content with the proportion of saturated acids in other milk fats and in stearic-rich animal depot fats (cf. Hilditch, 1940). In this respect, therefore, the human-milk fat glycerides conform with the typical structure of those of the milk fats of other animals.

Component glycerides in the human-milk fat fractions A to D. With the aid of the information given above it is possible to deduce (*a*) the increments of each component acid present in each of the fractions A, B, C and D, and (*b*) therefrom to consider the possible combinations of glycerides present in each fraction, thus leading to an approximate estimate of the component glycerides of the complete human-milk fat.

Table 2 shows the increments (% by mol.) of each acid present in the fractions A, B, C and D, and also the combined increments of the acids grouped together respectively as decanoic-myristic, palmitic, stearic (with arachidic), oleic (with lower, minor mono-ethenoid acids), octadecadienoic, and unsaturated C_{20-22} acids.

Since the method of separation employed ensures to a considerable extent that, for example, fractions C and D will contain no fully saturated glycerides and that fraction D is unlikely to contain more than a minute proportion of mono-unsaturated glycerides, whilst, similarly, tri-unsaturated glycerides are wholly absent from fractions A and B and unlikely to be present in fraction C, and since the fully saturated glycerides in A and B have been determined in detail, it is permissible to consider

Table 2. *Component acids (increments % by mol.) in fractions A, B, C and D*

	A		B		C	D	Total
Glycerides (% by mol.)	24·0		19·0		21·3	35·7	100·0
Component acids (increments % by mol.):							
Decanoic	0·4		0·2		0·3	1·2	2·1
Lauric	1·6		1·5		1·4	3·2	7·7
Myristic	2·7		2·2		2·2	1·9	9·0
Palmitic	9·6		6·5		5·0	2·5	23·6
Stearic	3·4		1·8		1·1	0·2	6·5
Arachidic	0·5		0·2		0·2	—	0·9
Decenoic	—		—		Trace	Trace	Trace
Dodecenoic	Trace		Trace		0·2	0·1	0·3
Tetradecenoic	Trace		0·1		0·2	0·5	0·8
Hexadecenoic	1·0		0·5		0·9	3·0	5·4
Oleic	3·9		5·0		7·7	16·6	33·2
Octadecadienoic	0·2		0·5		1·5	4·9	7·1
Unsaturated C_{20-22}	0·7		0·5		0·6	1·6	3·4
	F.S.G.*	Mixed	F.S.G.*	Mixed			
Component acid groups:	(7·8 %)	(16·2 %)	(1·3 %)	(17·7 %)			
C_{10}–C_{14} saturated	2·9	1·8	0·7	3·2	3·9	6·3	18·8
Palmitic	3·0	6·6	0·6	5·9	5·0	2·5	23·6
Stearic (+C_{20})	1·9	2·0	—	2·0	1·3	0·2	7·4
Oleic (+C_{10}–C_{16} unsaturated)	—	4·9	—	5·6	9·0	20·2	39·7
Octadecadienoic	—	0·2	—	0·5	1·5	4·9	7·1
Unsaturated C_{20-22}	—	0·7	—	0·5	0·6	1·6	3·4

* F.S.G. = fully saturated glycerides.

the various ways in which different acids may be calculated (from the data in Table 2) to be associated with the remaining acids in any one fraction. In Table 3 this is shown for the following combinations: (*a*) saturated with unsaturated acids, (*b*) palmitic with the rest of the acids ('others'), (*c*) oleic with the rest of the acids, and (*d*) linoleic (octadecadienoic) with the rest of the acids.

fats which have been submitted to the crystallization process supports this assumption, so that, although the data in Table 4 do not represent positive analytical isolation or determination of the components given, it is probable that the figures represent a near approximation to the true values for each component glyceride, or groups of component glycerides, mentioned. (In Table 4 the lower

Table 3. *Possible groupings of acids in component glycerides of fractions A, B, C and D*

	A		B				
	F.S.G.*	Mixed	F.S.G.*	Mixed	C	D	Total
Glycerides (% by mol.)	7·8	16·2	1·3	17·7	21·3	35·7	100·0
(*a*) Fully saturated	7·8	—	1·3	—	—	—	9·1
Mono-unsaturated-disaturated	—	14·7	—	15·4	9·5	—	39·6
Di-unsaturated-mono-saturated	—	1·5	—	2·3	11·8	27·1	42·7
Tri-unsaturated	—	—	—	—	—	8·6	8·6
(*b*) Dipalmito-mono-'others'	1·1	3·5	0·6	—	—	—	5·2
Monopalmito-di-'others'	6·7	12·7	0·7	17·5	15·0	7·5	60·1
Tri-'others'	—	—	—	0·2	6·3	28·2	34·7
(*c*) Dioleo-mono-'others'	—	—	—	—	1·8	14·0	15·8
Monooleo-di-'others'	—	11·8	—	15·1	19·5	21·7	68·1
Tri-'others'	7·8	4·4	1·3	2·6	—	—	16·1
(*d*) Monolinoleo-di-'others'	—	0·7	—	1·5	4·5	14·8	21·5
Tri-'others'	7·8	15·5	1·3	16·2	16·8	20·9	78·5

* F.S.G. = fully saturated glycerides.

When the possible groupings of the acids in the human-milk fat glycerides (Table 3) are considered in relation to the individual component acids in each respective fraction (A, B, C and D), the more detailed statement of component glycerides shown in Table 4 follows: the figures in Table 4 depend to some extent upon the general assumption that, in the relatively simple mixtures obtained by the preliminary crystallization procedure, the glycerides are assembled so as to produce the maximum formation of 'mixed' glycerides. Experience which has now been gained over a wide variety of natural

saturated acyl groups, myristic, lauric, and decanoic, are grouped together as 'C_{10-14}', and all the unsaturated derivatives are considered together as 'unsaturated'.)

DISCUSSION

The data in the final column of Table 4 indicate that nearly half of the human-milk fat consists of glycerides in which two of the acyl groups are unsaturated; in this part of the fat, about 24% (of the whole fat) also contains, as saturated acyl group, myristic, lauric or decanoic acid, and about 19%,

Table 4. *Probable component glycerides of human-milk fat*

	A		B				
	F.S.G.*	Mixed	F.S.G.*	Mixed	C	D	Total
Glycerides (% by mol.)	7·8	16·2	1·3	17·7	21·3	35·7	100·0
Fully saturated (9·1%):							
Di-C_{10-14}-monopalmitin	1·0	—	0·7	—	—	—	1·7
Mono-C_{10-14}-dipalmitin	1·1	—	0·6	—	—	—	1·7
Mono-C_{10-14}-palmitostearin	5·7	—	—	—	—	—	5·7
Mono-unsaturated-disaturated (39·6%):							
Mono-'unsaturated'-C_{10-14}-palmitin	—	5·3	—	9·4	5·6	—	20·3
Mono-'unsaturated'-C_{10-14}-stearin	—	—	—	0·2	1·7	—	1·9
Mono-'unsaturated'-dipalmitin	—	3·5	—	—	—	—	3·5
Mono-'unsaturated'-palmitostearin	—	5·9	—	5·8	2·2	—	13·9
Di-unsaturated-monosaturated (42·7%):							
Mono-C_{10-14}-di-'unsaturated'	—	—	—	—	4·6	19·0	23·6
Palmito-di-'unsaturated'	—	1·5	—	2·3	7·2	8·1	19·1
Tri-unsaturated (8·6%)	—	—	—	—	—	8·6	8·6

* F.S.G. = fully saturated glycerides.

palmitic acid. In addition, about 9% of the fat consists of tri-unsaturated glycerides. Of the total fatty acids in the fat, 33% is oleic and 17% is made up of the minor unsaturated acids, and it may be significant that there is just sufficient of the latter to account for these di- and tri-unsaturated glycerides being wholly constituted as mono-oleo-mono-saturated and di-oleo-glycerides respectively, the remaining acyl group in each case being contributed by one or other of the minor unsaturated acids. If this interpretation be correct, oleic acid is almost the only unsaturated component of the mono-unsaturated-disaturated glycerides. As mentioned earlier, this view also accords with the specific distribution of linoleic acid as revealed by the data in Table 1.

The lower saturated acids are seen to be distributed throughout the milk fat in combination with (*a*) two unsaturated acid groups, (*b*) oleic and palmitic, (*c*) palmitic and stearic, (*d*) oleic and stearic, or (*e*) one or two palmitic acid groups. With the two di-unsaturated groups already mentioned, mono-C_{10-14}-oleopalmitins (20%), and oleopalmitostearins (14%) are in fact the only major component glycerides of the fat. These features are probably of some importance in relation to the mode of origin of the lower saturated acid components; they are fundamentally similar to those subsisting in cow-milk fats, where the range of lower saturated acids extends further, and down to butyric acid. This general similarity in component glycerides between the two milk fats is illustrated in Table 5, which gives a summary of the total component acids of each fat, together with the proportions of glycerides containing (*a*) one lower saturated group, (*b*) two lower saturated groups, (*c*) one palmitic, and (*d*) two palmitic groups (i) in the human-milk fat and (ii) in a cow-milk fat (Hilditch & Paul, 1940).

Table 5. *Comparative glyceride structure of human- and cow-milk fats*

	Human	Cow
Component acids (% by mol.):		
C_{14} and lower (saturated)	18·8	27·4
Palmitic	23·6	21·1
Stearic (+arachidic)	7·4	10·6
C_{16} and lower (mono-ethenoid)	6·5	4·1
Oleic	33·2	31·4
Octadecadienoic	7·1	4·9
Unsaturated C_{20-22}	3·4	0·5
Component glyceride groups (% by mol.):		
(*a*) Glycerides containing 1 C_{14} (or lower) saturated group	53	*c.* 50
(*b*) Glycerides containing 2 C_{14} (or lower) saturated groups	2	*c.* 15
(*c*) Glycerides containing 1 palmitic group	60	*c.* 65
(*d*) Glycerides containing 2 palmitic groups	6	*c.* 5

Owing to the greater number of acidic components in cow-milk fat, the numerical data for the component glyceride groups could not be computed so definitely as in the present instance, but the figures in Table 5 show that there is close similarity in the values for the respective groups of glycerides, having regard to the varying proportions, in the fats as a whole, of the acids concerned. The palmitic glycerides are elaborated on similar lines in human- and in cow-milk fats, about 80% or more of the total palmitic acid being in the form of mono-palmito-glycerides. The lower saturated acids also occur predominantly in combination with two acyl radicals of different character (palmitic, oleic, etc.), the proportion of glycerides containing two of these lower saturated groups increasing, however, in the cow-milk fat in which the acids in question form 27% of the total acids (as compared with 19% in the human-milk fat).

These general resemblances in the glycerides of the two milk fats support the view suggested in our previous communication (Hilditch & Meara, 1944, p. 32), namely, that the mode of formation of the lower fatty acids may well be the same in both cases, but that the process proceeds much more extensively, and to a lower range of acids, in the production of cow-milk fat than in that of human-milk fat. One of us has suggested (Hilditch & Sleightholme, 1930, 1931) that the source of the characteristic milk and depot fats of animals may be a preformed mixture of oleo-glycerides in which an unsaturated group is changed on the one hand into a short-chain acyl group, or on the other hand reduced to a stearo-glyceride. In this connexion reference should be made to the recent studies of human depot (subcutaneous) fat by Cramer & Brown (1943), who found, *inter alia*, the following component acids in the case of a female human-body fat: lauric 0·1, myristic 2·7, palmitic 24·0, stearic 8·4, tetradecenoic 0·2, hexadecenoic 5·0, oleic 56·9, octadecadienoic (mainly linoleic) 10·2, and unsaturated C_{20-22} acids 2·5% (by wt.). The resemblance to human-milk fat in the palmitic acid content is obvious; further, just as the lower saturated acids are less abundant in human- than in cow-milk fat, so also is there less production of stearic acid in the human depot fat as compared with ox depot fats.

More adequate discussion of this view might be possible if more data for the component acids of the blood glycerides of animals were available. At present there is only the very interesting observation of Kelsey & Longenecker (1941), who found that the component acids of ox-blood glycerides included palmitic 33·7, stearic 22·2, hexadecenoic 2·6, oleic 21·3, linoleic 18·4 and unsaturated C_{20-22} acids 1·1% (by mol.). The blood glycerides of the cow, from which the milk glycerides are now

recognized to be derived (Graham, Jones & Kay, 1936; Maynard, McCay, Ellis, Hodson & Davis, 1938; Shaw & Petersen, 1938; Shaw, 1939), thus contain the same or similar proportions of palmitic acid as in the depot and milk fats, a content of stearic acid similar to the depot fats, and unsaturated C_{18} acids which differ from both the corresponding depot and milk fats in that nearly half is made up of linoleic or *cis-cis*-$\Delta^{9,12}$-octadecadienoic acid, which is substantially absent from both depot and milk fats of the cow and other ruminants. If the observations of Kelsey & Longenecker (1941) should later be found to be characteristic for animal-blood glycerides in general, the possibility would be opened that the components of blood glycerides which are the source of the short-chain saturated acids in milk fats are linoleo-glycerides rather than oleo-glycerides as first suggested. This view is attractive both because linoleo-groups would be expected on general grounds to be more reactive and susceptible to metabolic change than oleo-groups, and because it accounts for parallelisms between the amount of short-chain acid formation and the amount of linoleic acid in the two milk fats. In cow-milk fat the production of the short-chain acids is very prominent, but only traces at most of linoleic acid (the *cis-cis*-form) are present, whilst in human-milk fat the short-chain acids only extend to C_{10} (instead of C_4), and their amount is much less, but the fat still contains a significant proportion of linoleic glycerides. Such hypotheses must nevertheless remain speculative until the results of more detailed analyses of the blood glycerides of different animals are made available, a field of enquiry which, although difficult, is clearly of considerable importance.

The suggestion has been made to us that the presence of linoleic glycerides in the human-milk fats which we have studied might be connected with the ingestion of these glycerides in the form of margarine, since our material was in all cases obtained from English mothers on war-time diets. We believe that this cannot be the source of the linoleic glycerides of the milk fats, however, since the earlier study of Bosworth (1934) on human-milk fat from American subjects (whose fatty diet must have been butter rather than margarine) showed exactly similar proportions of linoleic acid therein. Linoleic acid thus appears normally to form 7% or thereabouts of the component acids of human-milk fat.

SUMMARY

1. Human-milk fat glycerides consist of about 24% of di-unsaturated glycerides in which the mono-saturated group is myristic, lauric or decanoic acid, about 19% of palmito-di-unsaturated glycerides, about 20% of glycerides containing one radical each of palmitic acid, unsaturated acid, and one of the C_{10}, C_{12} or C_{14} acids, about 14% of mono-unsaturated-palmitostearins, and minor proportions of various other mixed glycerides.

2. The proportions of mono-palmito-glycerides and of glycerides containing one radical of either of the lower saturated acids have close resemblances to the proportions of the corresponding groups of glycerides in cow-milk fats, and thus suggest that the origin of the short-chain glycerides may be the same in both fats.

3. It is probable that the di- and tri-unsaturated glycerides of human-milk fat contain one radical of linoleic or other unsaturated acid, the remainder being oleic, and that the mono-unsaturated glycerides are almost wholly mono-oleo-glycerides. It is also possible that the source of milk-fat short-chain glycerides may be linoleo-glycerides, rather than oleo-glycerides as previously suggested.

We are deeply indebted, as in the previous work, to Dr S. K. Kon, of the National Institute for Research in Dairying, Shinfield, for his assistance in obtaining for us sufficient human-milk fat to enable the present work to be undertaken.

REFERENCES

Bosworth, A. W. (1934). *J. biol. Chem.* **106**, 235.
Cramer, D. L. & Brown, J. B. (1943). *J. biol. Chem.* **151**, 427.
Graham, W. R., Jones, T. S. G. & Kay, H. D. (1936). *Proc. Roy. Soc.* B, **120**, 330.
Hilditch, T. P. (1940). *The Chemical Constitution of Natural Fats*, p. 238, Fig. 4. London: Chapman and Hall, Ltd.
—— & Meara, M. L. (1944). *Biochem. J.* **38**, 29.
—— & Paul, S. (1940). *J. Soc. chem. Ind., Lond.*, **59**, 138.
Hilditch, T. P. & Sleightholme, J. J. (1930). *Biochem. J.* **24**, 1098.
—— —— (1931). *Biochem. J.* **25**, 507.
Kelsey, F. E. & Longenecker, H. E. (1941). *J. biol. Chem.* **139**, 727.
Maynard, L. A., McCay, C. M., Ellis, G. H., Hodson, A. Z. & Davis, G. K. (1938). *Mem. Cornell agric. Exp. Sta.* no. 211.
Shaw, J. C. (1939). *J. Dairy Sci.* **22**, 438.
—— & Petersen, W. E. (1938). *J. Dairy Sci.* **21**, 122.

The Component Acids of Milk Fats of the Goat, Ewe and Mare

By T. P. HILDITCH, *Department of Industrial Chemistry, University of Liverpool*, AND H. JASPERSON, *Research Department, Messrs J. Bibby and Sons, Ltd., Liverpool*

(*Received* 1 *September* 1944)

In marked contrast to the fairly extensive range of published information on the fatty acid composition of cow-milk fats, there have appeared relatively few detailed analyses of the milk fats of other farm animals. Dhingra (1933) analyzed the milk fats of Indian goats and sheep by the ester fractionation procedure, using a Willstätter flask, and found them to be closely similar in composition. The two fats differed from cow-milk fat in the higher content of hexanoic, octanoic and decanoic acids and in the lower content of unsaturated C_{18} acids. Riemenschneider & Ellis (1936) made an investigation of goat-milk fat and reported the presence of monoethenoid C_{10}, C_{14} and C_{16} acids. Investigation by Laxa (1927, 1931) of the milk fat of the mare and of the sow led to a deduction of very approximate proportions of the major constituent acids; and, very recently, de la Mare & Shorland (1944) have published a few more data of the major acids of sow-milk fat.

The present communication deals with fractionation analyses, using an efficient heated column, of the methyl esters of fats obtained from samples of milks of goat, ewe and mare.

METHODS

The component acids in the three milk fats were determined by separation and ester fractionation as described by Hilditch & Jasperson (1941). In the case of the mare-milk fat, a smaller column (12 in. by $\frac{1}{2}$ in.) was used, instead of that described by Longenecker (1937), for distillation of the small amount of the steam volatile acids and of the esters of ethanol-insoluble lead soaps of the non-volatile acids.

The goat's milk was supplied by Mrs G. W. Howard, King's Lynn, Norfolk, from does on a daily feed of hay, kale, beet, 1–1½ lb. groundnut cake, and also 2 hr. pasture; the ewe's milk was sent by Prof. J. G. Wright of the School of Veterinary Science, University of Liverpool; and the mare's milk was obtained from a Shire mare, 8 years old, belonging to Mr H. C. Peers, Oakland Farm, Neston, Cheshire, which was fed on pasture in summer and on hay with a small amount of crushed oats in winter.

RESULTS

General characteristics. The general characteristics of the three fats are given in Table 1 (*a*), and in Table 1 (*b*) are added some values reported by other investigators.

The data confirm the evidence of earlier workers that goat- and sheep-milk fats have somewhat similar general characteristics; and that they have lower iodine values and higher Polenske values than cow-milk fats.

It would appear that whilst mare-milk fat is also characterized by a relatively high Polenske value, there is a notable difference between this fat on the one hand and goat- and sheep-milk fats on the other, in respect of the low Reichert-Meissl and Kirschner values and the high iodine value of the former.

Table 1. *Characteristics of goat-, ewe- and mare-milk fats*

	Saponification equiv.	Iodine value	Reichert-Meissl value	Polenske value	Kirschner value
		(*a*) Present series			
Goat	233·7	28·8	23·2	7·2	15·6
Ewe	244·5	36·7	26·2	3·6	17·6
Mare	253·1	84·3	6·2	5·9	2·6
		(*b*) Data reported by other workers			
Goat (Dhingra, 1933)	238·1	28·8	31·7	8·2	19·9
Goat (Knowles & Urquahart, 1924)	—	24·7–36·9	24·5–27·8	4·9–8·7	16·8–19·0
Ewe (Dhingra, 1933)	232·4	32·1	33·9	8·0	22·8
Ewe (Faltin & Dedinszky, 1930)	—	—	22·5–39·4	2·2–6·9	15·8–21·0
Mare (Laxa, 1927)	246·3	61·0	—	6·1	—

Component fatty acids. The percentages (by wt. and mol.) of the component acids of the three fats are given in Table 2 and are arranged for comparison in Table 3.

Table 2. *Component acids of milk fats*

	Steam-volatile acids	Non-steam-volatile: 'Solid' acids	Non-steam-volatile: 'Liquid' acids	Total	Fatty acids (% by wt.)	Fatty acids (% by mol.)
Goat	(8·9%)	(43·4%)	(47·7%)			
Butyric	2·98	—	—	2·98	3·0	7·5
Hexanoic	2·45	—	—	2·45	2·5	4·7
Octanoic	1·36	—	1·44	2·80	2·8	4·3
Decanoic	1·55	—	8·41	9·96	10·0	12·8
Lauric	0·38	1·13	4·46	5·97	6·0	6·6
Myristic	—	7·58	4·63	12·21	12·3	11·8
Palmitic	—	25·24	2·58	27·82	27·9	24·1
Stearic	—	5·97	—	5·97	6·0	4·7
As Arachidic	—	0·64	—	0·64	0·6	0·4
Decenoic	—	—	0·25	0·25	0·3	0·3
Dodecenoic	—	0·01	0·24	0·25	0·3	0·3
Tetradecenoic	—	0·07	0·76	0·83	0·8	0·8
Hexadecenoic	—	0·72	1·85	2·57	2·6	2·2
Oleic	0·18	2·01	18·86	21·05	21·1	16·5
Octadecadienoic	—	—	3·56	3·56	3·6	2·8
Unsaturated C_{20-22}	—	—	0·25	0·25	0·2	0·2
Unsaponifiable	—	0·03	0·41	0·44	—	—
Ewe	(9·1%)	(45·4%)	(45·5%)			
Butyric	2·83	—	—	2·83	2·8	7·5
Hexanoic	2·57	—	—	2·57	2·6	5·3
Octanoic	2·16	—	—	2·16	2·2	3·5
Decanoic	0·77	—	3·98	4·75	4·8	6·4
Lauric	0·50	0·41	2·94	3·85	3·9	4·5
Myristic	—	5·79	3·88	9·67	9·7	9·9
Palmitic	—	20·45	3·39	23·84	23·9	21·6
Stearic	—	12·52	—	12·52	12·6	10·3
As Arachidic	—	1·10	—	1·10	1·1	0·8
Decenoic	—	—	0·13	0·13	0·1	0·2
Dodecenoic	—	0·01	0·12	0·13	0·1	0·2
Tetradecenoic	—	0·05	0·50	0·55	0·6	0·6
Hexadecenoic	—	0·55	1·69	2·24	2·2	2·0
Oleic	0·23	4·52	21·46	26·21	26·3	21·6
Octadecadienoic	—	—	5·21	5·21	5·2	4·3
Unsaturated C_{20-22}	—	—	1·85	1·85	1·9	1·3
Unsaponifiable	0·04	—	0·35	0·39	—	—
Mare	(6·4%)	(18·1%)	(75·5%)			
Butyric	0·39	—	—	0·39	0·4	1·1
Hexanoic	0·88	—	—	0·88	0·9	1·9
Octanoic	2·45	—	0·13	2·58	2·6	4·4
Decanoic	1·39	—	4·08	5·47	5·5	7·9
Lauric	—	—	5·52	5·52	5·6	6·8
Myristic	—	3·42	3·43	6·85	7·0	7·4
Palmitic	—	10·46	5·44	15·90	16·1	15·4
Stearic	—	2·81	—	2·81	2·9	2·4
As Arachidic	—	0·26	—	0·26	0·3	0·2
Decenoic	—	—	0·87	0·87	0·9	1·3
Dodecenoic	—	—	0·99	0·99	1·0	1·2
Tetradecenoic	—	0·10	1·67	1·77	1·8	1·9
Hexadecenoic	—	0·39	6·98	7·37	7·5	7·2
Oleic	1·29	0·52	39·97	41·78	42·4†	36·9†
Octadecadienoic	—	—	(−3·8H*)	(−3·7H*)		
Linolenic	—	—				
Unsaturated C_{20-22}	—	—	5·05	5·05	5·1	4·0
Unsaponifiable	—	0·14	1·37	1·51	—	—

* H = atoms of hydrogen (per molecule) short of saturation.

† The unsaturated C_{18} acids have been shown, by application of the method of alkali-induced conjugation, to consist of 44% octadecenoic, 18% octadecadienoic and 38% octadecatrienoic acid (cf. p. 446).

Table 3. *Comparison of component acids of goat-, ewe- and mare-milk fats*

Acid	Goat	Ewe	Mare
	Percentages by weight		
Butyric	3·0	2·8	0·4
Hexanoic	2·5	2·6	0·9
Octanoic	2·8	2·2	2·6
Decanoic	10·0	4·8	5·5
Lauric	6·0	3·9	5·6
Myristic	12·3	9·7	7·0
Palmitic	27·9	23·9	16·1
Stearic	6·0	12·6	2·9
As Arachidic	0·6	1·1	0·3
Decenoic	0·3	0·1	0·9
Dodecenoic	0·3	0·1	1·0
Tetradecenoic	0·8	0·6	1·8
Hexadecenoic	2·6	2·2	7·5
Oleic	21·1	26·3	18·7
Octadecadienoic	3·6	5·2	7·6
Linolenic	—	—	16·1
Unsaturated C_{20-22}	0·2	1·9	5·1
	Molar percentages		
Butyric	7·5	7·5	1·1
Hexanoic	4·7	5·3	1·9
Octanoic	4·3	3·5	4·4
Decanoic	12·8	6·4	7·9
Lauric	6·6	4·5	6·8
Myristic	11·8	9·9	7·4
Palmitic	24·1	21·6	15·4
Stearic	4·7	10·3	2·4
As Arachidic	0·4	0·8	0·2
Decenoic	0·3	0·2	1·3
Dodecenoic	0·3	0·2	1·2
Tetradecenoic	0·8	0·6	1·9
Hexadecenoic	2·2	2·0	7·2
Oleic	16·5	21·6	16·3
Octadecadienoic	2·8	4·3	6·6
Linolenic	—	—	14·0
Unsaturated C_{20-22}	0·2	1·3	4·0

DISCUSSION

The proportions of butyric acid show close parallelism with the Reichert-Meissl and Kirschner values given in Table 1. The percentages are similar in goat- and ewe-milk fats and somewhat lower than in typical milk fat from stall-fed cows. The low butyric acid content (1·1 % by mol.) is an arresting feature of the short-chain acids of mare-milk fat, and corresponds to the low Kirschner value (2·6). It is generally accepted that comparative indications of the relative proportions of butyric acid are given by the Kirschner value, of butyric and hexanoic acids by the Reichert-Meissl value, and of octanoic and decanoic acids by the Polenske value.

The relatively high content of decanoic acid in goat- and sheep-milk fats, found by Dhingra (1933), has been generally confirmed in the present work, but a point of difference is seen from the fact that, whereas the present goat-milk fat contained 12·8 % and the sheep-milk fat contained 6·4 % decanoic acid, the decanoic acid contents of the Indian milk fats were very similar, namely 11·1 and 10·1 % respectively.

The milk fat of the horse resembles those of the goat and sheep in being characterized by a relatively higher decanoic acid content than cow-butter fat: in fact, all three fats contain somewhat higher percentages of C_8, C_{10} and C_{12} acids than are found in the cow-milk fats recorded in the literature (cf. Hilditch, 1940).

With regard to the percentage of stearic acid in the three fats, it will be noted from the data in Table 3 that the ewe-milk fat (10·3 % by mol. stearic acid) contains more than twice the percentage found in the goat-milk fat (4·7 % by mol.) and four times that in the mare-milk fat (2·4 % by mol.): in this respect the milk fat of the ewe resembles that of the cow.

Another interesting difference between the three fats is the relatively lower percentage (15·4) of palmitic acid in the mare-milk fat as compared with the more usual percentage (20–30) in goat-, sheep- and cow-milk fats. It is noteworthy, however, that mare-milk fat contains a corresponding relatively higher amount of hexadecenoic acid, namely 7·2 % instead of about 2 % in the other milk fats. Thus, the total C_{16} acids approach near the average range of about 25–30 % which apparently is characteristic of milk fats of most mammals so far studied.

The lower saturated acids from butyric to myristic as given in Tables 2 and 3 show goat-milk fat to contain 47·7 %, ewe-milk fat 37·1 % and mare-milk fat 29·5 %. The high content in the goat-milk fat is caused mainly by the presence of higher proportions of decanoic and myristic acids; and the relatively lower figure for mare-milk fat results mainly from the small component proportions of butyric and hexanoic acids.

The unsaturated C_{18} acids of mare-milk fat. The outstanding difference in the present series of analyses from those previously recorded for milk fats of herbivorous mammals emerged from a study of the component unsaturated C_{18} acids, and in particular those of the mare-milk fat. Iodine value determinations of the methyl esters from the ethanol-soluble lead salts of the non-steam-volatile acids of this fat indicated that the mean unsaturation of the C_{18} acids was considerably higher than of those of the goat-, ewe- or cow-milk fat, namely, i.v. 164·0 for mare-milk C_{18} acids as compared with the more usual iodine values of 90–100.

A portion (2·11 g.) of the mare-milk fat C_{18} esters was hydrolyzed and the resulting acids were brominated in ether at 0° to give 0·78 g. insoluble bromides, m.p. 178–180° (recrystallized from benzene, m.p. 181°; mixed m.p. 181° with authentic specimen of hexabromostearic acid). This yield corresponded to 13·6 % 'α'-linolenic acid and would be indicative of about 40 % linolenic acid in the C_{18} unsaturated acids if the hexabromide ratios of Shinowara & Brown (1938) were applied. Another

portion (2·05 g.) was brominated in light petroleum (40–60°) at 0° to yield 0·87 g. insoluble bromides melting at 166–170°. Pure hexabromostearic acid, m.p. 181°, was readily isolated from this mixture but no tetrabromostearic acid, m.p. 114°, was obtained.

The proportion of octadecadienoic and octadecatrienoic acids present in methyl ester fractions which consisted wholly of esters of unsaturated C_{18} acids has been determined by a modification of the method of Mitchell, Kraybill & Zscheille (1943) which will be described elsewhere by one of us. Briefly, the basis of the method is isomerization by alkali under standardized conditions of the mixed acids, which results in the production of definite proportions of conjugated diethenoid acids from linoleic acid, and of conjugated di- and tri-ethenoid acids from linolenic acid or from their geometric isomers. The conjugated acids give characteristic bands in the ultraviolet spectrum respectively at 234 and 268 mμ. From the extinction coefficients of these bands the amounts of the two acids may be calculated. Application of this method in the present instance indicated that the unsaturated C_{18} acids in the 'liquid' acids of mare-milk fat consisted of 38 % octadecatrienoic, 18 % octadecadienoic and 44 % octadecenoic acid.

Thiocyanometric analyses suggested the presence of about 44·5 % of triethenoid acid, with consequent absence of diethenoid acid. So far as our experience goes at the moment, it is believed that the new spectroscopic method is the most reliable at present available for the analysis of mixtures of oleic, linoleic and linolenic acids or esters, or their geometric isomers. We consider that the proportions of the three acids indicated by the spectroscopic analysis are probably close to the truth, and it will be noted that the amount of octadecatrienoic acid (38 %) accords with the figure of about 40 % linolenic acid deduced above from the crystalline hexabromostearic acid obtained on bromination.

The presence of linolenic acid in mare-milk fat is of considerable interest and shows a notable difference from the composition of cow-milk fat under similar conditions of feeding. The mare in question was on a pasture diet during the summer months when it was being foaled, and hence the feeding conditions were somewhat analogous to those of cows on summer pasture recorded in the investigations of Hilditch & Sleightholme (1930), Hilditch & Dean (1933) and Hilditch & Jasperson (1941). Although the lipids of pasture probably contain about 40 % linolenic acid (Smith & Chibnall, 1932; Shorland, 1944), none of this acid was found in the milk fat of the cows, and furthermore, none has been isolated from ox depot fats (Hilditch & Longenecker, 1937). Perhaps similar factors which lead to the presence of a small proportion (2 %) of linolenic acid in the body fat of the horse (Heiduschka & Steinrock, 1921) also influence the appearance of this acid in the milk fat.

The goat- and sheep-milk fat unsaturated C_{18} acids appear to resemble in nature those of the cow-milk fat: the point of difference lies in the lesser content (by about 10 % by mol.) of oleic acid. The mixed unsaturated C_{18} acids of the goat-milk fat were subjected to low temperature crystallization as described previously for the cow-milk fat C_{18} acids (Hilditch & Jasperson, 1941). The resulting polyethenoid concentrate, i.v. 123, was brominated at 0° in ether and in light petroleum (40–60°). No evidence for the presence of 'α'-linoleic acid was obtained, and it was concluded that the main polyethenoid constituent is the octadecadienoic acid of uncertain constitution also found in the cow-milk fat.

The above data have been ascertained from the milk fats of animals which may be considered to be influenced by the same factors as shown by Hilditch and co-workers to operate in the case of the cow, for instance, diet, state of lactation, age of the animal, degree of exercise and seasonal temperatures. In view of the fact that the mare-milk fat has not been hitherto analyzed in any detail, the results recorded for this fat in the present paper should be considered as tentative until definite conclusions can be drawn when an opportunity has occurred to study further specimens.

SUMMARY

1. Detailed analyses of the fatty acid composition have been determined of the milk fats of the goat, ewe and mare.

2. The general similarity between goat- and ewe-milk fats has been confirmed; and differences have been indicated by the relatively higher content of decanoic and a relatively lower content of stearic acid in goat-milk fat than in ewe-milk fat.

3. Mare-milk fat, which has not been analyzed in detail hitherto, has been shown to comprise the following acids (% by mol.): butyric 1·1, hexanoic 1·9, octanoic 4·4, decanoic 7·9, lauric 6·8; myristic 7·4, palmitic 15·4, stearic 2·4, as arachidic 0·2, decenoic 1·3, dodecenoic 1·2, tetradecenoic 1·9, hexadecenoic 7·2, oleic 16·3, octadecadienoic 6·6, linolenic 14·0, unsaturated C_{20-22} 4·0. The most arresting features are the low butyric and hexanoic acid contents and the presence of substantial proportions of linolenic acid amongst the C_{18} unsaturated acids.

We are indebted to Prof. J. G. Wright, Mrs R. G. Howard, Mr H. C. Peers and Miss E. Thomas for placing the experimental material at our disposal; to Mr F. Burke and Miss E. H. Harrop for valuable assistance in the ester fractionation analyses; to Mr A. McKerrigan for determination of the general analytical characteristics of the fats; and to the Directors of Messrs J. Bibby and Sons, Ltd., for permission to publish the results of the investigation.

REFERENCES

Dhingra, D. R. (1933). *Biochem. J.* **27**, 851.
Faltin, A. & Dedinszky, G. (1930). *Rep. Hung. agric. Exp. Sta.* **33**, 62.
Heiduschka, A. & Steinrock, A. (1921). *J. prakt. Chem.* (2), **102**, 241.
Hilditch, T. P. (1940). *Chemical Constitution of Natural Fats.* London: Chapman and Hall, Ltd.
—— & Dean, H. K. (1933). *Biochem. J.* **27**, 889.
—— & Jasperson, H. (1941). *J. Soc. chem. Ind., Lond.*, **60**, 305.
—— & Longenecker, H. E. (1937). *Biochem. J.* **31**, 1805.
—— & Sleightholme, J. J. (1930). *Biochem. J.* **24**, 1098.
Knowles, F. & Urquahart, J. C. (1924). *Analyst*, **49**, 509.
Laxa, O. (1927). *Ann. Falsif., Paris*, **20**, 462.
—— (1931). *Ann. Falsif., Paris*, **24**, 87.
Longenecker, H. E. (1937). *J. Soc. chem. Ind., Lond.*, **56**, 199T.
de la Mare, P. B. D. & Shorland, F. B. (1944). *Nature, Lond.*, **153**, 380.
Mitchell, J. H., Kraybill, H. R. & Zscheille, F. P. (1943). *Industr. Engng Chem.* (Anal. ed.), **15**, 1.
Riemenschneider, R. W. & Ellis, N. R. (1936). *J. biol. Chem.* **113**, 219.
Shinowara, H. Y. & Brown, J. B. (1938). *J. Amer. chem. Soc.* **60**, 2734.
Shorland, F. B. (1944). *Nature, Lond.*, **153**, 168.
Smith, J. A. B. & Chibnall, A. C. (1932). *Biochem. J.* **26**, 218.

The Stabilization and Estimation of Pyruvic Acid in Blood Samples

By C. LONG, *Department of Biochemistry, The Queen's University, Belfast*

(*Received* 21 *September* 1944)

Two methods are available for assessing the aneurin (vitamin B_1) nutritional level of the body. The first of these involves the determination of aneurin itself in blood or urine, especially after test doses, and has met with only partial success. (For a review of the literature, see Bicknell & Prescott, 1942.) The other procedure depends upon the fact that pyruvic acid, an intermediary in carbohydrate metabolism, is utilized by the tissues at a rate which is largely determined by their co-carboxylase (aneurin pyrophosphoric ester) content; thus when dietary aneurin is inadequate and the tissues contain suboptimal amounts of co-carboxylase, pyruvate is only slowly metabolized and its concentration in the blood tends to increase.

While pyruvate is readily estimated in protein-free filtrates (Long, 1942), the concentration of pyruvic acid present *in vivo* in the blood is not easily determined since its value falls during the period following the drawing of the blood sample. Lu (1939) and Friedemann & Haugen (1942) overcame this difficulty by deproteinizing the blood specimen as quickly as possible after withdrawal from the vein. Bueding & Wortis (1940), however, did not find this method satisfactory, but claimed to have stabilized the level of blood pyruvate for a few minutes by means of iodoacetate. More recently, Golberg & Gillman (1943) have shown that in the presence of both iodoacetate and fluoride the pyruvate level remains constant for 30 min.

The aim of the present investigation has been to find some method of stabilizing, for a longer period, the pyruvate level of a blood sample at its initial value. This would allow the postponement of an estimation until a sufficient number of samples had accumulated; specimens might also be sent by post. This aim has been so successfully achieved that blood samples may now be kept at room temperature for at least 3 weeks without variation in the pyruvate level. The method of stabilization requires haemolysis at a slightly acid reaction in the presence of fluoride.

FACTORS CONTROLLING CHANGES IN THE PYRUVIC ACID LEVEL OF SHED BLOOD

Materials

Na *pyruvate*. Prepared according to Peters (1938). A solution containing a known amount (about 5μg.) in 2 ml. 10% trichloroacetic acid was used as a standard.

Na *iodoacetate*. Iodoacetic acid was neutralized with 40% NaOH to pH 7·5. Final concentration in blood, 0·2–2·0%.

Citrate buffers of suitable pH values. Citric acid monohydrate (mol. wt. 210) (0·840 g.), dissolved in about 1·5 ml. water, was treated with 40% NaOH till the required pH was attained. One-fifth of the total volume was then pipetted into a tube and evaporated to dryness (see Methods (*a*)); this amount was used for each 10 ml. blood sample, giving a final concentration of 0·08M.

Haemolytic agents. 'White' saponin (F. E. Becker and Co.) and thymol were used at final concentration 0·5 and 0·25% respectively. Cetyltrimethylammonium bromide ('Cetavlon') in solid form (75%) and as a 7·5% solution was obtained from Imperial Chemical (Pharmaceuticals) Ltd. Final concentration 0·3%.

All other reagents were analytical specimens, the final concentration in blood being: oxalic acid (0·05M), K oxalate (0·02 and 0·01M), Na citrate (0·02M) and NaF (0·1M).

Blood from normal male subjects was used.

Methods

(*a*) *Pretreatment of tubes to contain blood samples.* Anticoagulants, with or without inhibitors, buffers and haemolytic agents, were introduced into test-tubes (graduated at 10 ml.) either as solids or in solution; in the latter case, the contents of the tubes were evaporated to dryness *in vacuo* on a boiling water-bath. The tubes were fitted with white rubber bungs, corks being unsuitable in view of the colorimetric procedure which followed, especially when the blood was acidified.

(*b*) *Collection of blood samples.* A 50 ml. syringe was filled with venous blood, a process lasting about 1 min. Each tube was then filled to the 10 ml. mark and the contents vigorously shaken for about 10 sec. In general, the procedure up to this point lasted not more than 2 min. This was considered satisfactory in view of the observation of Friedemann & Haugen (1942) that the pyruvate level of untreated blood samples remains constant for 3 min.

(*c*) *Preparation of protein-free filtrates.* 1 ml. blood was slowly pipetted drop by drop, to ensure maximum drainage, into 4 ml. 12·5% trichloroacetic acid and the mixture thoroughly shaken. The tube was allowed to stand 20 min. before filtration. When a high blood pyruvate level was expected, less than 1 ml. blood was taken.

(*d*) *Pyruvic acid content of the filtrate.* The estimation was made on 2 ml. protein-free filtrate, the method being that of Long (1942) without modification. Briefly this depends on the conversion of the pyruvic acid into its 2:4-dinitrophenyl-hydrazone, followed by extraction of the latter first into ethyl acetate solution and then into aqueous Na_2CO_3; finally the Na_2CO_3 extract was treated with excess NaOH and the intensity of the red-brown colour developed was determined photometrically. Blank determinations with 2 ml. 10% trichloroacetic acid were always simultaneously performed and the accuracy of the method was frequently checked using Na pyruvate standards.

(*e*) *Recovery of pyruvic acid.* A blood sample was found to contain 1·01 mg. pyruvic acid/100 ml. Pyruvic acid (as Na pyruvate) was added to the sample in amount equivalent to 0·50 mg./100 ml. Total pyruvic acid was determined and found to be 1·515 mg./100 ml. Difference, 0·505 mg.; recovery, 101%.

(*f*) pH *of blood samples.* In those experiments in which the blood was acidified with citrate buffer, the final pH of the sample was often determined. The author wishes to thank Prof. D. C. Harrison for making these determinations with the glass electrode apparatus.

Results

The effect produced by the anticoagulant. Wilkins, Weiss & Taylor (1938) first showed the rapid disappearance of pyruvate from oxalated blood, an observation which has been confirmed by Bueding & Wortis (1940). A destruction of pyruvate under similar conditions has also been observed in the present investigation (Table 1).

When citrate is used as anticoagulant, however, the rate of pyruvate disappearance during the first 30 min. is much reduced (Table 2) as compared with oxalate. Moreover, this difference in behaviour of the two anticoagulants is even greater when the experiment is continued for some days (Table 3).

Table 1. *Disappearance of pyruvic acid from oxalated blood*

0·02M-oxalate present.

Time (min.)	Pyruvic acid (mg./100 ml.)
2·2	0·91
3·3	0·86
4·5	0·78
7·6	0·69
13·4	0·47
21·6	0·36
31·6	0·28

Table 2. *Pyruvate disappearance from oxalated and citrated blood*

Time (min.)	Pyruvic acid (mg./100 ml.)	
	0·02M-oxalate	0·02M-citrate
2·7	0·83	—
5·3	—	0·81
7·8	0·63	—
12·7	—	0·77
15·0	0·46	—
21·4	0·31	—
27·1	—	0·73

Table 3. *Blood pyruvate level after some days at room temperature*

Time (min.)	Pyruvic acid (mg./100 ml.)	
	0·02M-oxalate	0·02M-citrate
3·0	0·70	—
7·4	0·56	—
10·1	—	0·65
16·5	0·43	—
18·6	—	0·63
(days)		
1	0·48	0·93
2	0·39	1·48
3	0·23	6·70
4	0·34	15·7
8	4·08	17·6

Thus, after standing for 24 hr., citrated blood shows a definite increase in pyruvate concentration over the initial value and this effect continues for about a week; in oxalated blood, on the other hand, the pyruvate concentration continues to fall during the first 3 days and then only slowly begins to rise. This increase in pyruvate concentration is not due to bacterial action, since identical figures have been observed in the presence of 0·5% phenol.

With oxalate 0·02M has been found to be more effective than 0·01M in preventing this rise in pyruvate concentration (Table 4).

The effect of fluoride. The experiment in Table 3 indicates that two separate reactions, one destroying and another producing pyruvic acid, may take place

Table 4. *The effect of oxalate concentration on the disappearance of pyruvate from blood*

Time (min.)	Pyruvic acid (mg./100 ml.) 0·01 M-oxalate	0·02 M-oxalate
3·6	0·58	—
6·9	—	0·45
10·4	0·26	—
13·8	—	0·26
21·4	0·25	—
29·1	—	0·24
(days)		
1	0·24	0·24
2	0·22	0·13
3	0·18	0·11
4	1·55	0·23
5	2·92	0·92
8	5·70	3·41

in shed blood. The most likely reactions concerned are respectively:

(i) Pyruvate + triosephosphate ⇌ lactate + 3-phosphoglycerate,

(ii) 3-Phosphoglycerate ⇌ 2-phosphoglycerate ⇌ phosphopyruvate → pyruvate.

Support for reaction (i) is found in the iodoacetate inhibition of pyruvate disappearance (Bueding & Wortis, 1940), in the approximate equivalence of pyruvate disappearance and lactate production in the presence of NaF (Bueding & Goodhart, 1941) and in the relevant observations of Dische (1936–7), Lu & Needham (1939) and Harris & Elgert (1941).

There is also much evidence in favour of reaction (ii). First, oxalate is known to inhibit the dephosphorylation of phosphopyruvate in muscle extracts (Lohmann & Meyerhof, 1934). This accords well with the observation of Friedemann & Haugen (1942) that during the first few minutes after withdrawal from the vein, pyruvate disappears rapidly from oxalated as compared with untreated blood.

Table 5. *Inhibition of pyruvate formation by fluoride*

Time (min.)	Pyruvic acid (mg./100 ml.) 0·02 M-oxalate	0·02 M-oxalate + 0·1 M-NaF	Time (min.)	Pyruvic acid (mg./100 ml.) 0·02 M-citrate	0·02 M-citrate + 0·1 M-NaF
6·6	—	0·81	4·1	—	0·91
9·0	0·79	—	7·8	1·04	—
11·5	—	0·70	11·2	—	0·59
13·8	0·66	—	15·4	0·97	—
21·4	—	0·45	23·1	—	0·57
25·2	0·37	—	31·7	0·92	—
33·5	—	0·37	35·7	—	0·56
(days)			(days)		
1	0·39	0·32	1	2·92	0·55
3	0·29	0·24	2	12·0	0·52
7	1·72	0·28	3	14·6	0·51
10	4·49	0·46			

Thus, in oxalated blood, pyruvate is utilized by reaction (i), its formation by reaction (ii) being largely inhibited; reaction (ii), on the other hand, readily takes place in 0·02 M-citrated blood and partly compensates for the pyruvate utilization by reaction (i) during the first 30 min., so that a slower rate of pyruvate disappearance is observed. As would be expected, 0·02 M-oxalate has proved to be more effective than 0·01 M-oxalate as an inhibitor of pyruvate formation by reaction (ii) (Table 4).

Further evidence for reaction (ii) is to be found in the well-marked inhibition of pyruvate formation in the presence of NaF (Table 5), though, as might be expected, this is most clearly shown with citrated blood where the reaction is not masked by oxalate inhibition. NaF is well known to inhibit the conversion of 2-phosphoglyceric to phosphopyruvic acid (Lohmann & Meyerhof, 1934).

The use of iodoacetate for stabilizing blood pyruvate. Bueding & Wortis (1940), in an attempt to inhibit the oxido-reduction between pyruvate and triosephosphate in shed blood, collected their samples in a syringe containing iodoacetate; concentrations from 0·2 to 2·0 % were effective in preventing the disappearance of pyruvate during the first few minutes. It cannot be said, however, that stabilization was achieved, since the published figures show a slow upward trend in the level of the blood pyruvate, the value after 30 min. having risen by 3–20 %. This behaviour has been amply confirmed by Golberg & Gillman (1943). It now became of interest to ascertain how blood samples treated with iodoacetate would behave on standing for longer periods. In Table 6 typical experiments with three concentrations of iodoacetate are recorded.

Considering first the effect of 1 % iodoacetate (Table 6 (*a*)), it will be seen that after standing for a day the blood pyruvate level has fallen considerably, indicating that at this concentration iodoacetate does not inhibit oxido-reduction completely; after standing for a few more days, however, the pyruvate concentration again begins to rise, showing that the breakdown of phosphoglycerate is becoming marked. 0·2 % iodoacetate (Table 6 (*b*)) shows the same behaviour; 2 % iodoacetate, on the other hand, has proved to be effective in inhibiting the dismutation for some days. An unsatisfactory feature of iodoacetate inhibition, however, has been pointed out by Friedemann & Haugen (1942), who observed that the initial blood pyruvate level was higher in the presence of iodoacetate than in its absence; in Table 6 (*b*) it may be noted that the initial level is higher in the presence of 2 % as compared with 0·2 % iodoacetate.

Golberg & Gillman (1943) added NaF (approx. 0·1 M) as well as Na iodoacetate to their blood samples and claimed that under these conditions the pyruvate concentration was stabilized for

Table 6. *The effect of iodoacetate, with and without fluoride, on the blood pyruvate level*

0·02M-oxalate present.

(*a*) 1% Na iodoacetate

Time (min.)	Pyruvic acid (mg./100 ml.) Iodoacetate absent	1% iodoacetate	1% iodoacetate, 0·1M-NaF
9·0	0·79	—	—
13·8	0·66	—	—
16·4	—	1·35	—
18·8	—	—	1·31
25·2	0·37	—	—
27·7	—	1·39	—
30·7	—	—	1·46
(days)			
1	0·39	0·76	0·65
4	0·27	0·87	0·69
7	1·72	1·04	0·83
10	4·50	1·31	0·96

(*b*) 0·2 and 2% Na iodoacetate

Time (min.)	Pyruvic acid (mg./100 ml.) Iodoacetate absent	0·2% iodoacetate	0·2% iodoacetate, 0·1M-NaF	2% iodoacetate	2% iodoacetate, 0·1M-NaF
6·9	0·59	—	—	—	—
10·1	—	0·78	—	—	—
15·9	0·30	—	—	—	—
19·1	—	—	0·76	—	—
22·1	—	—	—	0·98	—
25·1	—	—	—	—	0·98
30·0	0·18	—	—	—	—
(days)					
1	0·12	0·38	0·22	1·21	0·43
4	1·53	0·32	0·16	—	0·55
7	6·30	1·09	0·52	1·20	0·63

30 min. at a level about 10% higher than the initial value. These conditions have also been examined in the present work (Table 6), and it will be seen that during the first few days the pyruvate level falls at all iodoacetate concentrations and later rises, approaching the initial value again after about a week. In view of the results obtained, the iodoacetate method has not been further investigated.

The pyruvate level as a function of the reaction of the blood. Since the two reactions involving pyruvate in shed blood, viz. oxido-reduction with triosephosphate and dephosphorylation of phosphopyruvate, both require enzyme systems, it was considered that a change of pH from neutrality might lead to enzyme inactivation and perhaps therefore to a stabilization of the blood pyruvate level. Encouraging results have been obtained on acidifying the blood sample. Table 7 compares the effect of 0·05M-Na oxalate and 0·05M-oxalic acid. During the first 30 min. the oxido-reduction is completely inhibited and, on standing, the breakdown of phosphopyruvate is largely prevented.

Table 7. *Effect of acidity on the blood pyruvate level*

Time (min.)	Pyruvic acid (mg./100 ml.) 0·05M-Na oxalate	0·05M-oxalic acid
11·8	0·81	—
14·2	—	0·98
20·4	0·60	—
22·0	—	0·99
29·9	0·49	—
31·6	—	0·99
(days)		
1	0·42	0·84
2	0·34	0·86
5	6·15	1·03

Further experiments of a more detailed nature have also been carried out with 0·08M-citrate buffers of known pH (Table 8); these buffers have a very suitable pH range and at the same time serve as anticoagulant. In this particular experiment, the final pH of the blood was determined roughly on a small sample of the plasma, with indicators. It will be noted that the pH 4 and 5 buffers are most effective in reducing the reaction rates. Unfortunately it was not possible to use buffers more acid than pH 4 since partial precipitation occurred, rendering the blood unsuitable for pipetting.

Table 8. *Blood pyruvate levels at different degrees of acidity*

pH of 0·08M-citrate buffer used	7·5	6·0	5·0	4·0
Final pH of mixture...	7·5	7·1	6·1	5·2
Time (min.)	Pyruvic acid (mg./100 ml.)			
3·6	0·87	—	—	—
6·2	—	1·14	—	—
9·1	—	—	1·08	—
12·1	0·73	—	—	—
15·1	—	—	—	0·99
18·0	—	1·17	—	—
20·7	—	—	1·10	—
23·7	0·68	—	—	—
26·8	—	—	—	0·93
(days)				
1	1·18	6·31	2·29	0·96
2	11·8	6·01	2·19	1·17
3	19·0	2·87	1·67	1·35
4	18·4	2·80	1·60	1·43

The effect of haemolysis on the blood pyruvate level. Haemolysis of blood samples was investigated for two reasons. First, it is often found that enzyme systems contained in red cells, and bound in some way to the structure of the cell, lose most of their activity when dispersed by haemolysis. Secondly, it seemed likely that in experiments conducted at low pH, the acid would more easily inactivate the enzymes in the absence of a cell structure. Harris & Elgert (1941) have shown that the enzyme system

causing the destruction of pyruvic acid in shed blood is contained within the red cells and further that cell suspensions haemolyzed by saponin or by freezing and thawing lose their ability to utilize pyruvate after 1 hr. at room temperature.

Different haemolytic agents have been employed in the present work with varying success. In an early experiment, crystalline thymol, finely powdered in a mortar, proved very satisfactory as a haemolytic agent and stabilized the blood pyruvate level for 4 days in the presence of a pH 4 buffer (Table 9). It will also be observed that, at neutral pH, the breakdown of phosphoglycerate appears to be completely inhibited by thymol haemolysis.

Table 9. *Haemolysis by finely powdered thymol for stabilizing blood pyruvate at an acid reaction*

pH of 0·08 M-citrate buffer used ...	7·5	7·5	4·0
	Pyruvic acid (mg./100 ml.)		
Time (min.)	Thymol absent	0·25% thymol	0·25% thymol
2·9	0·59	—	—
3·8	—	—	0·69
11·0	—	0·44	—
14·2	—	—	0·74
21·0	0·36	—	—
26·6	—	—	0·71
29·7	—	0·30	—
(days)			
1	0·33	0·27	0·73
2	5·12	0·22	0·74
3	14·3	0·21	0·74
4	16·8	0·21	0·73

Unfortunately, a very great disadvantage associated with thymol haemolysis was the fact that the blood samples after standing some days both at neutrality and at acid pH lost their homogeneity and contained particles in suspension which necessitated the use of a pipette with a tip of wide bore. For this reason, therefore, experiments were made using saponin as haemolytic agent. These, however, were not successful, for it appeared that even the best procurable samples of 'white' saponin contained a yellow-brown pigment which interfered with the colorimetric procedure; the extent of interference increased with the time the blood was kept.

In an attempt to find a more suitable haemolytic agent, various substances were examined, including cetyltrimethylammonium bromide ('Cetavlon'), which is used clinically as a detergent. This compound has proved to be an excellent haemolytic agent, the red cells being laked immediately by an amount equivalent to a final concentration of 0·3%. Also it does not suffer from the disadvantage noted above in the case of saponin, being quite colourless. Table 10 shows the effect of 'Cetavlon' at different pH values.

Table 10. *Blood pyruvate levels in the presence of 'Cetavlon' as haemolytic agent*

0·3% 'Cetavlon' present.

pH of 0·08 M-citrate buffer used	7·5	6·0	5·0	4·0
Final pH of mixture...	7·5	7·35	6·3	5·4
Time (days)	Pyruvic acid (mg./100 ml.)			
0	0·95	0·97	0·96	1·00
1	0·58	1·01	0·97	0·97
2	0·51	0·98	0·96	1·00
3	0·52	0·92	1·02	1·00
4	0·57	0·80	1·03	1·01
21	2·11	1·78	1·74	1·23

It will be noted that the blood pyruvate level remains constant for 3–4 days with a pH 4 or 5 buffer, for 2 days only with a pH 6 buffer and not at all at neutrality. In the case of blood treated with a pH 4 buffer, the possibility of stabilizing the pyruvate level for longer periods by adding NaF suggested itself. This procedure was attended with great success and appears to be a most reliable method of stabilizing pyruvic acid in blood samples.

DETAILS OF THE PROCEDURE FOR THE STABILIZATION AND ESTIMATION OF PYRUVIC ACID IN BLOOD

Citric acid monohydrate (168 mg.), dissolved in about 0·5 ml. distilled water, is treated with 40% NaOH until pH 4 is reached. One-half the volume of this citrate buffer is pipetted into a test-tube. The buffer is then evaporated to dryness *in vacuo* on a boiling water-bath. To the dry residue are added 21 mg. NaF and 20 mg. 'Cetavlon' (75%). The tube is then fitted with a white rubber bung. The contents of the tube appear to be quite stable; no deterioration has been found when the tube has been allowed to stand for a few weeks.

Blood is withdrawn from a vein in the ante-cubital fossa into a 5 ml. syringe, transferred to the collecting tube and shaken thoroughly. By this means the blood pyruvate level is stabilized.

For the estimation of pyruvic acid, 1 ml. of the blood sample is pipetted drop by drop into 4 ml. 12·5% trichloroacetic acid and vigorously shaken. After 20 min. the mixture is filtered through a 7 cm. filter paper (Whatman no. 1), about 3 ml. filtrate being obtained. 2 ml. of the protein-free filtrate are pipetted into a test-tube. Two other test-tubes are taken, one containing 2 ml. of a solution of standard Na pyruvate (about 5 μg.) in 10% trichloroacetic acid and the other containing 2 ml. 10% trichloroacetic acid (blank experiment). The rest of the procedure is carried out according to the method of Long (1942). The amount of pyruvic acid found in 2 ml. protein-free filtrate is multiplied by

Table 11. *Stabilization of the blood pyruvate level by 'Cetavlon'-haemolysis in the presence of citrate buffer, pH 4, and fluoride*

Time (days)	Pyruvic acid (mg./100 ml.)	Time (days)	Pyruvic acid (mg./100 ml.)
0	1·15	7	1·16
1	1·15	10	1·18
2	1·18	14	1·13
3	1·12	21	1·14
4	1·15		

250 to give the pyruvic acid concentration in mg./100 ml. blood.

In an experiment using 10 ml. blood, the pyruvate level has been found to be quite constant for at least 3 weeks, as shown in Table 11. This pyruvate level was identical with that observed when the blood sample, in the absence of 'Cetavlon', was precipitated immediately with trichloroacetic acid.

SUMMARY

1. The changes in pyruvic acid level of blood samples treated with different anticoagulants, inhibitors and haemolytic agents have been investigated. The results obtained are in harmony with the occurrence of the two following reactions:

(i) Pyruvate + triosephosphate ⇌ lactate + 3-phosphoglycerate,

(ii) 3-Phosphoglycerate ⇌ 2-phosphoglycerate ⇌ phosphopyruvate → pyruvate.

2. The detailed procedure for stabilizing and estimating pyruvic acid in blood samples is given. Stabilization is brought about by haemolysis with cetyltrimethylammonium bromide in the presence of fluoride and a pH 4 citrate buffer; the estimation is by means of the 2:4-dinitrophenyl-hydrazone.

REFERENCES

Bicknell, F. & Prescott, F. (1942). *The Vitamins in Medicine.* London: Heinemann.

Bueding, E. & Goodhart, R. (1941). *J. biol. Chem.* **141**, 931.

—— & Wortis, H. (1940). *J. biol. Chem.* **133**, 585.

Dische, Z. (1936–7). *Enzymologia*, **1**, 288.

Friedemann, T. E. & Haugen, G. E. (1942). *J. biol. Chem.* **144**, 67.

Golberg, L. & Gillman, T. (1943). *S. Afr. J. med. Sci.* **8**, 117.

Harris, J. S. & Elgert, S. E. (1941). *Proc. Soc. exp. Biol., N.Y.*, **47**, 321.

Lohmann, K. & Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.

Long, C. (1942). *Biochem. J.* **36**, 807.

Lu, G. D. (1939). *Biochem. J.* **33**, 249.

—— & Needham, D. M. (1939). *Biochem. J.* **33**, 1544.

Peters, R. A. (1938). *Biochem. J.* **32**, 2031.

Wilkins, R. W., Weiss, S. F. & Taylor, F. L. H. (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 296.

Preparation of Penicillin. Improved Method of Isolation*

By J. C. CLAYTON, B. A. HEMS, F. A. ROBINSON, R. D. ANDREWS and R. F. HUNWICKE,
Glaxo Laboratories Ltd., Greenford, Middlesex

(*Received* 24 *January* 1944; *deposited with the Royal Society* 23 *May* 1944–23 *September* 1944)

Fleming (1929) noticed that a mould which had accidentally contaminated a plate culture of *Staphylococcus aureus* was surrounded by a clear ring of agar, which suggested that the mould had produced an antibiotic substance in its immediate neighbourhood. He made no attempt, however, to isolate this substance, to which he gave the name penicillin, and the first attempt to do so was made three years later by Clutterbuck, Lovell & Raistrick (1932). They confirmed Fleming's observations that penicillin was active against Gram-positive but not against Gram-negative organisms, and showed that it could be extracted by ether from aqueous solutions at pH 2. They gave details for growing the mould, now known as *Penicillium notatum* Westling, on a synthetic medium of Czapek-Dox type, instead of the broth media used by Fleming.

Widespread interest in penicillin was aroused by two papers published by Florey and his colleagues (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941; Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing & Sanders, 1940), who described a method of preparing crude concen-

* This paper describes work completed by the end of 1942. Its publication has been delayed for security reasons. The authors are aware that considerable progress has been made in the preparation and isolation of penicillin since this preliminary work was carried out.

trates of penicillin from the metabolism solution produced by the growth of *P. notatum*, and showed that the product was of value in the treatment of certain infective conditions. These papers also described the bacteriostatic and bactericidal properties of penicillin, *in vitro* and *in vivo*, and the effects of serum and other biological materials on the activity. The effects of penicillin on leucocytes and tissues were also described, together with results of absorption and excretion experiments in man and laboratory animals.

The method of isolation now described differs radically from that recommended by the Oxford workers; it is more convenient and more efficient for large-scale working, and has been carried out on a substantial scale.

EXPERIMENTAL

Cultivation of the mould

Selection of strain. The first stage of the investigation comprised the selection of the best of nine different strains of *P. notatum* provided by Prof. H. Raistrick. The moulds were grown on a modification, introduced by Dr Turley, of the well-known Czapek-Dox medium; this had the following composition:

$NaNO_3$	3·0 g.	$FeSO_4.7H_2O$	0·01 g.
KH_2PO_4	1·0 g.	Glucose	40·0 g.
KCl	0·5 g.	Distilled water to	1000 ml.
$MgSO_4.7H_2O$	0·5 g.		

The solutions were decanted from the mould mycelia and tested for antibacterial activity against *Staphylococcus*, *Streptococcus* and *Pneumococcus*. From these preliminary experiments, several of the strains were selected for further examination, and the results indicated the marked superiority of strain B 592, originally obtained in 1929 from the Medical Research Council (National Institute for Medical Research).

Maintenance of stock cultures of Penicillium notatum. Stock cultures of *P. notatum* strain B 592 were grown on several different media, including Czapek-Dox agar, Czapek-Turley agar, Czapek-Dox agar containing sucrose instead of glucose, and malt or wort agar. It was found that those cultures grown on malt or wort agar gave the most reliable and morphologically stable moulds. The malt agar was made from a 5% solution of commercial malt extract and the wort agar from concentrated wort (sp. gr. 1·040). These stock cultures were held for at least 3 months before being subcultured, and it has been our custom throughout to inoculate our production batches from fresh subcultures prepared from an old stock culture, the parent culture being used for as long as possible, until growth was exhausted. Care was taken, in making these subcultures, to transfer only the spores, avoiding transfer of the mycelium as far as possible, for it was found that mycelial cultures tended to give poor yields of penicillin. The subcultures, like the stock cultures, were made on malt agar slopes, which were incubated at 24° and used for sowing the batches when 7 days old.

Cultivation of the mould. The medium used for production of penicillin was Turley's modification of the Czapek-Dox medium described above. In the experimental stage of the work, 400 ml. portions were introduced into 1 l. conical flasks, which were autoclaved for ½ hr. at a pressure of 15 lb./sq.in. It is known that autoclaving a solution of this type causes changes in the glucose, and the rotation of the autoclaved medium corresponded with about 2·5% instead of 4% of glucose; the copper reduction figure was equivalent to 4%. A suspension of spores, made by introducing 10 ml. of Czapek-Turley medium into each of the subcultures and dislodging the spores with a sterile stainless steel spatula, was used for inoculating the flasks, the contents of one culture tube being distributed as evenly as possible among four flasks, which were then shaken and incubated in the dark at 24°. Subsequently, however, it was found to be quicker and more convenient to transfer the spores directly from the slopes by means of a moistened platinum loop.

The growth of the mould was characteristic and has not varied greatly over a long period; only occasionally has serious trouble been experienced through a falling off in the yields of penicillin. The above precautions have, however, been rigidly followed; it is especially important to use a subculture obtained from an old stock culture. After 2–3 days the surface of the liquid in the flasks became covered with a white felt; this gradually formed folds, the crests of which were blue-green; orange-yellow transpiration drops accumulated in these folds. The reverse side was golden yellow in colour, and the colour of the solution gradually deepened to an intense yellow. Penicillin production was complete in 10–14 days. During this time the apparent concentration of glucose fell steadily, the optical rotation reaching a minimum value corresponding to about 0·5%. The pH of the solution fell to about 4 in the first 4–5 days and then rose to 7·0–7·6.

The method of assay

The testing of penicillin was carried out by the serial dilution method (Clutterbuck *et al.* 1932). Each test solution was sterilized by filtration through a small Seitz filter, and dilutions were made in tubes containing 4 ml. of digest broth medium of pH 7·2, the series forming a geometrical progression from 1 in 10 to 1 in 2560. Each tube of medium was

then sown with one loopful of a 24 hr. culture of the chosen *Staphylococcus aureus* strain, and the results were read after incubation for 18 hr. at 37°.

The medium was a papain-digest broth, employed by us for other purposes, and was made by mixing 1000 g. of minced horse muscle with 4 l. of distilled water and digesting the suspension with 10–15 g. of papain at 65–70° for 3 hr., the pH being maintained at 7·0 throughout. The digest was filtered, made alkaline to phenolphthalein and sterilized by steaming for 1 hr. on two successive days. The concentrated digest was filtered to remove precipitated phosphates, and then diluted with an equal volume of normal saline. The pH was adjusted to 7·4, and the solution tubed and autoclaved at a pressure of 20 lb./sq.in.

The strain of *Staph. aureus* used was isolated in these laboratories from a pathological specimen, and stock cultures of it were carried on agar. Test cultures were made from these by transference to the papain-digest broth and incubation for 24 hr., the culture so obtained then being used for inoculating the penicillin solution, and 0·1 ml. of the suspension being added to each 4 ml. tube.

Inoculations with a drawn-out pipette were made from a 24 hr. culture in the same medium. Once a week the culture was plated out on blood agar. Addition of glucose enhanced the growth in papain-digest broth, but the end-point was less sharply defined. Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942) showed that notatin, another substance produced by *Penicillium notatum*, inhibits the growth of *Staphylococcus aureus* if the medium used contains glucose; in view of this it is essential that the medium used in testing metabolism solutions for penicillin should not contain glucose.

An interchange of samples with other workers has shown that it is possible to obtain good agreement between assay results from different laboratories. Occasional discrepancies have been encountered, however, and recently the practice has been adopted of comparing each batch of samples with a standard preparation.

Variations in composition of medium

The amount of penicillin produced in early batches was very variable, the dilutions necessary to inhibit the growth of *Staphylococcus* ranging from 1 in 200 to 1 in 1600 for different batches, and even for different flasks of the same batch. Moreover, if the metabolism solutions were allowed to stand, marked loss of activity occurred in a few days.

Stability of penicillin at various pH's. It was found that the solutions were more stable, and indeed showed very little loss after 14 days, if they had been boiled for 10 min. and then cooled. This is illustrated by an experiment in which a metabolism solution was tested at intervals and the activity compared with that of a solution which had been boiled for 10 min. The results are shown in Table 1. This increased stability was possibly due to the destruction of an enzyme capable of inactivating penicillin.

The stability of penicillin in metabolism solutions was found to be optimal at pH 7–8, and little or no activity was lost when such solutions were kept at 80° for 2 hr. A solution of pH 5, on the other hand, lost all its activity when heated at 60° for 4 hr., whilst a solution of pH 3 lost all its activity at room temperature (23°) in 1 day and in 3 days at 0°. Solutions of pH 10 and above were also unstable; most of their activity was lost on storage at room temperature for 3 days.

Table 1. *Effect of boiling the metabolism solution on the stability of penicillin during storage*

Pretreatment of solution	Titre of solution after storage for (days) 0	3	7	14
None	1/1280	1/160	1/40	—
Boiled for 10 min.	1/1280	1/1280	1/1280	1/640

Modifications of medium for penicillin production. Towards the end of 1941, after the formation of the Therapeutic Research Corporation Ltd., we continued this work in collaboration with the Wellcome Physiological Research Laboratories (W.P.R.L.), where a new medium for growing the mould had been elaborated by Dr C. G. Pope. This consisted of the Czapek-Turley medium with added copper sulphate, sodium acetate and sodium citrate, and had the following composition: glucose, 4 %; $NaNO_3$, 0·4 %; KH_2PO_4, 0·1 %; KCl, 0·05 %; $MgSO_4.7H_2O$, 0·05 %; $FeSO_4.7H_2O$, 0·01 %; sodium citrate, 0·2 %; sodium acetate, 0·2 %; $CuSO_4.5H_2O$, 0·00175 %.

The B 592 strain on this medium gave more consistent yields of penicillin than on the Czapek-Turley medium, and the penicillin itself appeared to be more stable; titres averaging 1 in 640, with some variation on either side of this figure, were obtained.

The optimal depth of the medium in culture was found to be 3–4 cm. A greater depth of liquid gave lower titres, the rate of diffusion of oxygen from the atmosphere then being insufficient for complete fermentation, since the lower layers remained colourless, whereas the layer immediately below the mycelium was dark yellow in colour.

The effect of modifying the composition of the W.P.R.L. medium was tested by making up with tap water a basal medium containing 4 % glucose, 0·1 % KH_2PO_4, 0·05 % KCl, 0·05 % $MgSO_4.7H_2O$, 0·001 % $FeSO_4.7H_2O$, and adding varying amounts of $NaNO_3$, $CuSO_4$, sodium citrate and sodium acetate. The titres obtained after growing the mould on these media for 11–12 days are shown in Table 2.

These preliminary results indicated that (*a*) the addition of copper alone either to the Czapek-Dox or to the Czapek-Turley medium had no effect on the penicillin production or was even harmful; (*b*) the addition of citrate and acetate had a limited effect; and (*c*) improved results were obtained by adding copper sulphate, citrate and acetate together.

Table 2. *Effect on penicillin production of adding various supplements to the basal medium*

Additions to basal medium (%)				
$NaNO_3$	$CuSO_4.5H_2O$	Sodium citrate	Sodium acetate	Titre
0·4	0·00175	0·2	0·2	1 in 640
0·4	0·00175	0	0	1 in 160
0·3	0·00175	0	0	1 in 80
0·3	0	0·2	0·2	1 in 320
0·3	0	0	0	1 in 160

Later experiments threw some doubt on the beneficial effect of copper; it proved difficult to obtain conclusive evidence on this point, for the variations from flask to flask were of the same order as those encountered between media with and without added copper. The conclusion reached after a large number of experiments was that with strain B 592 the addition of copper increased the maximum titre slightly or not at all, but rendered the yields of penicillin more consistent; it undoubtedly increased the rate and degree of sporulation. The presence of copper also appeared to inhibit the formation of the yellow pigment chrysogenin, first described by Clutterbuck *et al.* (1932); only negligible amounts of this pigment were isolated from metabolism solutions containing copper + acetate + citrate.

The effect of other substances on the yield of penicillin and on the morphology of the mould was also studied. Tyrosine up to 0·045 % was without effect, but the addition of 0·01 % of asparagine markedly increased the concentration of penicillin produced during 11 days' incubation. Ascorbic acid (0·1 %) and nicotinic acid (0·1 %) were without effect on the titre, but the former produced a bluer felt and the latter increased the growth rate. Aneurin (0·01 %) and riboflavin (0·01 %) had little or no effect. *dl*-Aspartic acid (0·01 %) had no effect, but 0·1 % of glutamic acid speeded up growth and led to a somewhat higher titre.

The simultaneous addition of aneurin, riboflavin, nicotinic acid, ascorbic acid and pimelic acid had no effect on the titre, the pH or the rate of glucose consumption. Guanidine hydrochloride (0·001 %) had a marked effect on the appearance of the mould and resulted in the rapid formation of numerous transpiration drops, but did not materially increase the yield of penicillin. The effect of guanine and adenine on the titre was negligible, whilst the addition of uracil (up to 0·05 %) gave a darker metabolism solution without affecting the titre.

Isolation of penicillin from metabolism solutions

(1) *Extraction methods.* The first attempts to isolate penicillin from the mould metabolism solution (Czapek-Turley medium) were made by the method of Abraham *et al.* (1941), using extraction with amyl acetate at pH 2. Very troublesome emulsions were obtained, however, and aqueous extracts from the amyl acetate solutions were only slightly active. Better results were obtained by the ether extraction method of Clutterbuck *et al.* (1932).

Portions (50 ml.) of the solution were extracted with three 35 ml. portions of ether, and the ethereal extract, after concentration by distillation in the presence of water, was tested alongside the residual metabolism solutions. Extraction was optimal at pH 3, some loss of penicillin taking place at pH 2. Extraction was facilitated by acidification to pH 3·6 and filtration to remove protein and chrysogenin, as recommended by Clutterbuck *et al.* (1932). The resulting filtrate did not form emulsions as readily as did the original solution.

The ethereal extract obtained from this filtrate was extracted with dilute $NaHCO_3$, and the aqueous solution acidified and extracted with ether. The barium salt of penicillin was prepared by shaking this with a suspension of excess $BaCO_3$, separating off the aqueous phase, filtering and evaporating the filtrate in the frozen state. The solid so obtained inhibited growth at a dilution of 1 in 4×10^6, and a 60 % recovery of the original activity was obtained.

One disadvantage of this process was encountered when it was applied to metabolism solutions obtained from the W.P.R.L. medium. A considerable amount of acetic acid was extracted along with the penicillin, so that calcium acetate was present in the product, which was therefore less active per mg. than similar material obtained from Czapek-Turley medium.

(2) *Adsorption methods.* It was found that penicillin was quantitatively adsorbed from protein-free metabolism solutions of pH 2, 3, 4, 5 and 7 by activated charcoal or fuller's earth (20 g./l. of metabolism solution); the charcoal removed the colour as well as the antibacterial activity. Less charcoal, e.g. 10 g./l., did not remove the activity completely. The activity could be recovered quantitatively from the charcoal adsorbate by elution with 80 % or absolute ethanol, or with methanol, acetone or pyridine, but the degree of concentration effected by adsorption and elution varied according to the pH at which the adsorption was carried out.

This is illustrated by an experiment in which portions of the same metabolism solution, adjusted to various pH values, were adsorbed on charcoal (20 g./l.), and the titres and total solids of the ethanol eluates determined. The results (Table 3) indicated that the best pH for adsorption

Table 3. *Effect of alterations in the* pH *of metabolism solution on recovery of penicillin and of total solids from charcoal adsorbates by elution with ethanol*

pH	Titre	Total solids (g./l.)
3	1/160	1·2
4	1/320	1·15
5	1/320	1·05
6	1/320	0·75
7	1/320	0·75
8	1/160	0·75
9	1/160	0·65

was 6–7. When a volume of ethanol equal to one-half that of the metabolism solution was used, most of the activity was contained in the first eluate; the amount of penicillin present in the third eluate was negligible. Elution was almost, but not quite, complete with a volume of ethanol one-quarter that of the metabolism solution. Aqueous acetone (20%), recommended by the W.P.R.L. workers, was found to be as good an eluant as ethanol, but aqueous ether proved to be less satisfactory, only 50% of the activity being recovered when a volume equal to that of the original solution was used.

A new method of working up metabolism solution was based on these results. The solution was acidified to pH 3·6 and precipitated protein and pigment were filtered off. The filtrate was neutralized (pH 7), stirred with 2% of its weight of charcoal (Sutcliffe and Speakman no. 5) and filtered. The charcoal was eluted on the filter with a one-half volume of ethanol and the filtrate diluted with a one-tenth volume of water and evaporated under reduced pressure, great care being taken to prevent the internal temperature from rising above 20°. After acidification to pH 3, the aqueous solution was extracted three times with two-thirds its volume of ether, the combined ethereal extracts were shaken with a suspension of excess $BaCO_3$ and the lower layer was separated off, filtered and evaporated in the frozen state. The solid so obtained inhibited completely the growth of *Staphylococcus aureus* at a dilution of 1 in $0{\cdot}75 \times 10^6$ to 1 in $1{\cdot}5 \times 10^6$, and contained 20–40% of the activity of the original solution.

When this process was applied to 20 l. batches, the yields were considerably below 20%. It was then learned that Dr C. G. Pope at the W.P.R.L., using the same process, was obtaining much more satisfactory recoveries, and a comparison of the two methods showed that he added chloroform to the metabolism solution to prevent bacterial contamination. It was found that the addition of chloroform to our metabolism solution greatly facilitated the subsequent elution of activity from the charcoal adsorbate, whilst adsorption was in no way impaired. All the activity was then recovered from the charcoal by elution with a one-fifth volume of 20% acetone. Under these conditions, the charcoal adsorption method gave better yields of better material, needed less bulk of materials than extraction with ether or other solvent, and required less complicated plant. Moreover, when sodium acetate was used in the medium, it was advantageous to use charcoal adsorption because it did not result in contamination of the final product with calcium acetate or barium acetate, as did solvent extraction. Yields varied from 30 to 60%, and the material inhibited growth at dilutions of 1 in 3×10^6 to 1 in 5×10^6.

Adsorption occurred more easily when the metabolism solution was filtered through a bed of charcoal instead of being stirred with charcoal as in the original process. In this way not only was the amount of charcoal required reduced from 20 to 4 g./l., but elution of the adsorbate was easier; even aqueous ether gave complete recovery. The resulting penicillin preparation was more active, and barium salts which inhibited growth in a dilution of 1 in 15×10^6, equivalent to 300 Oxford units/mg., were obtained. This observation has not been confirmed by the W.P.R.L. workers, who obtained only partial elution under these conditions. With the strain of *Penicillin notatum* used at the W.P.R.L. we also failed to obtain complete extraction; we can only conclude that other strains of mould produce impurities which affect adsorption and elution.

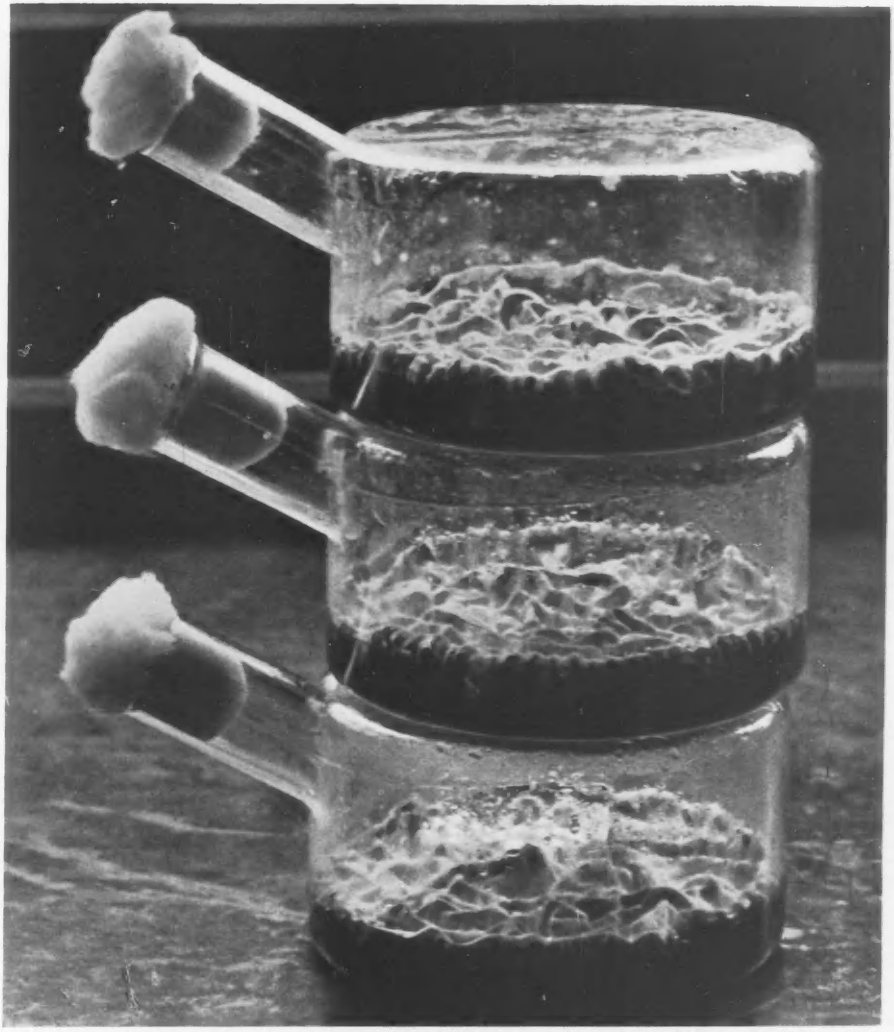
The effect of the presence of organic solvents and of inorganic salts in the aqueous solution on the adsorptive power of the charcoal was next studied. The penicillin was adsorbed when a solution saturated with ether was run through a bed of charcoal, but a solution containing in addition 1–2% of NaCl retained its activity under the same conditions. No penicillin was then adsorbed but a considerable reduction in the colour was effected, with a corresponding increase in the activity of the resulting calcium salt from 100 to 140 units/mg. The process gave smaller total yields, however, when applied to large batches, and was discontinued.

Large-scale production of penicillin

The general procedure described above is now being operated on a fairly large scale. The mould is grown in special glass flasks (Pl. 3). These flasks are so designed that several can be stacked during incubation; for filling and sterilization they are accommodated in specially designed wire baskets, each of which holds 12 flasks. This reduces the necessity for frequent handling of individual flasks.

Each flask holds 800 ml. of W.P.R.L. medium, which is introduced by means of a large tap attached to a measuring device. The flasks are then plugged with cotton-wool and autoclaved for 10 min. at a pressure of 10 lb./sq.in. After cooling overnight, the flasks—still in the wire baskets—are inoculated by means of a wire loop with spores of *P. notatum* grown on a wort-agar slope, the inoculation being carried out in a closed cabinet. The flasks are then taken from the baskets and stacked on racks, which are wheeled into an incubator room maintained at a constant temperature of $25 \pm 1°$.

After 14 days' incubation, the liquid is filtered through muslin and then run through a bed of charcoal and kieselguhr (which facilitates filtration) prepared on a large stoneware funnel; 5 g. of charcoal are used for each litre of metabolism solution. When all the solution has filtered, the charcoal is washed with a little water and then saturated with chloroform. The penicillin is eluted by running through the charcoal a volume of 20% acetone equal to one-tenth that of the original metabolism solution. The eluate, acidified to pH 2·5–3·0 with 10% H_3PO_4, is emulsified by rapid stirring with an equal volume of chloroform, and the emulsion then broken by means of an Alfa-Laval separator. The chloro-



Photograph by Bertram Park

Mould culture flasks in use for penicillin production.

form layer is collected separately and stirred with 0·5 % $NaHCO_3$. Alternatively, the chloroform solution is shaken with a suspension of $CaCO_3$ to give a solution of calcium penicillin. Both the sodium salt and the calcium salt, obtained from the solutions by drying at low temperatures (between $-30°$ and $-15°$) *in vacuo*, are bright yellow amorphous powders, which generally inhibit growth at a dilution of about 1 in 10×10^6; this is equivalent to an activity of 200 Oxford units/mg.; 1000 l. of metabolism solution are produced and worked up each day, with an average titre of 15 Oxford units/ml., and 60–70 % of the total activity is recovered as sodium or calcium salt. With a somewhat smaller installation producing 200 l. of metabolism solution each day, yields of 75–80 % are obtained.

Attempted purification of crude penicillin

Various attempts were made to purify further the crude penicillin obtained from the metabolism solution by charcoal adsorption and elution.

(*a*) *Formation of salts of penicillin.* Several attempts were made to obtain sparingly soluble salts of penicillin. An aqueous solution of barium penicillin, on treatment with benzyl-*pseudo*thiuronium chloride, gave a yellow precipitate, whilst the filtrate contained all the activity of the original solution. Since the amount of precipitate formed was very small, however, the degree of purification effected was insufficient to justify the routine use of this procedure.

None of the following quaternary ammonium salts gave insoluble salts with penicillin: pyridinium and quinolinium methiodide, benzyl-quinolinium chloride, *p*-nitrobenzyl-pyridinium chloride and *m*-nitrophenyl-trimethyl ammonium iodide.

(*b*) *Miscellaneous.* Catch, Cook & Heilbron (1942), by means of their chromatographic method, in which columns of silica gel mixed with alkaline earth carbonates are used, succeeded in increasing the activity of some of our material, containing 200 units/mg., to over 1000 Oxford units/mg.

Treatment with aluminium amalgam, recommended by Abraham *et al.* (1941), was applied to our material and resulted in almost complete decolorization of the solution; all the activity of the original salt was found in the supernatant liquid, and some degree of concentration was effected. The necessity for employing in this connexion the troublesome three-stage chromatographic treatment recommended by Abraham *et al.*, and the greater success of the chromatographic method of Catch *et al.* (1942), led us to abandon this line of attack. Penicillin can also be reduced without loss of activity by catalytic hydrogenation in presence of platinum oxide, and by electrolytic reduction by means of a mercury cathode and lead anode with a diaphragm.

Attempts to improve the purity of penicillin by electrodialysis in a simple cell or by foaming according to the method of Schütz (1937, 1942) were unsuccessful.

Potency. The best specimens of penicillin prepared by us are inhibitory to *Staphylococcus aureus* at a dilution of 1 in 15×10^6 to 1 in 20×10^6, but are probably not more than 10–20 % pure; no useful purpose would be served by a description of their chemical and physical properties.

Stability. In aqueous solution the activity of crude penicillin is unchanged on standing for several days at pH 8, but 50 % of the activity is lost at pH 3·6 in 8 hr. at 20° or in 24 hr. at 0°. It is also destroyed by standing overnight in solution in ethanol or methanol.

The dry barium or calcium salt can be heated for 1 hr. at 100° *in vacuo* without loss of activity, though some of our batches proved unstable to heat and even to cool storage. An aqueous solution of the salts is inactivated by boiling for 10 min., in striking contrast to the stability of penicillin in metabolism solutions; the latter can be boiled in a copper-free medium (see above) for 10 min. without loss.

Penicillin solutions are inactivated by tin, zinc, lead and ferric salts, 0·01 % destroying the activity completely in 24 hr. at 0°. Copper has an even more striking effect, 0·001 % being sufficient to inactivate penicillin solutions in 24 hr. at 0°. Ferrous salts are comparatively harmless, whilst silver appears to be inert. The deleterious effect of copper was not inhibited by addition of 0·1 % glycine or citrate. The effect of copper is rather surprising, for, whereas the presence of up to 4·5 p.p.m. in the mould metabolism solution apparently does no harm, somewhat larger amounts, e.g. 10 p.p.m., are sufficient to inactivate purified preparations of penicillin in a few hours. The presence of varying amounts of copper or ferric iron in our calcium salts probably accounts for the different stability of different batches.

SUMMARY

1. A satisfactory method of cultivating *Penicillium notatum*, and a method of isolating crude penicillin from the metabolism solution, are described.

2. Penicillin can be adsorbed from aqueous solutions by activated charcoal and can be eluted from the adsorbate by certain organic solvents, or mixtures of solvents with water.

3. The production of preparations of penicillin containing up to 200 units/mg., by an adsorption and elution process, is now being carried out on a large scale, the mould being grown in specially designed glass flasks.

We thank the Directors of the Therapeutic Research Corporation Ltd. for permission to publish this account of our work, and we also wish to record our gratitude to Prof. H. Raistrick for the very considerable help he has given us throughout our investigations. We have to thank too our colleagues in the Therapeutic Research Corporation, especially Dr J. W. Trevan and Dr C. G. Pope of the Wellcome Physiological Research Laboratories, whose collaboration has been particularly valuable; their results have been made freely available to us and have greatly facilitated our work. Finally, we have to acknowledge the help given, in designing the flasks, by Mr A. G. Howkins, and in the laboratory work by Messrs G. V. Cawthorne, H. H. Somerville, and the Misses A. M. Hill, J. A. Withycombe and J. Waldie.

REFERENCES

Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G. & Jennings, M. A. (1941). *Lancet*, **2**, 177.
Catch, J. R., Cook, A. H. & Heilbron, I. M. (1942). *Nature, Lond.*, **150**, 633.
Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J. & Sanders, A. G. (1940). *Lancet*, **2**, 226.
Clutterbuck, P. W., Lovell, R. & Raistrick, H. (1932). *Biochem. J.* **26**, 1907.
Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1942). *Nature, Lond.*, **150**, 634.
Fleming, A. (1929). *Brit. J. exp. Path.* **10**, 226.
Schütz, F. (1937). *Nature, Lond.*, **139**, 629.
—— (1942). *Trans. Faraday Soc.* **38**, 85, 94.

A Critical Examination of Lugg's Method for the Determination of *l*-Ascorbic Acid. 2

By G. A. SNOW and S. S. ZILVA (Member of the Scientific Staff, Medical Research Council), *Division of Nutrition, Lister Institute, London*

(*Received* 11 *August* 1944)

Our previous contribution to the subject (Snow & Zilva, 1943) was devoted mainly to the study of the kinetics of the combination of pure ascorbic acid, reductic acid and reductone with HCHO, and the results were utilized for the consideration of the limitations of Lugg's method (1942). Further observations made us aware of the fact that certain indophenol-reducing substances which are formed on heating solutions containing glucose in the presence of alkali behaved differently in their combination with HCHO to pure reductone. It therefore became desirable to investigate in detail the problem that arose. The results which are reported in this communication, apart from raising points of theoretical interest, throw further light on the limitations of the method. A technique which we consider yields the most satisfactory results under the circumstances is described.

METHODS

Reagents

l-*Ascorbic acid and reductic acid* (see Snow & Zilva, 1941).
Reductone (see Snow & Zilva, 1938).
Formaldehyde (see Snow & Zilva, 1943).
Metaphosphoric acid. Laboratory reagent. Solutions prepared freshly each day.
Pectin. Commercial specimen prepared from lemons. Completely soluble in water, giving an almost colourless opalescent solution. pH of 1% w/v solution = 3·04. Free acid—equivalent to 0·98 ml. N-NaOH/g. (indicator—phenolphthalein).
Glycine. Analar reagent twice recrystallized from water.
Ethylaminoacetate hydrochloride. Prepared from Analar glycine and recrystallized from ethanol; m.p. 143°.
Methyleneaminoacetonitrile. Dimeric form prepared by the method of Klages (1903); twice recrystallized from ethanol; m.p. 129°. The molar concentrations of this compound given in this paper are calculated on the basis of the monomeric formula.
Methylamine. Dilute solutions prepared from a 33% aqueous solution. Concentration determined by titration with HCl.
Alanine (dl). Synthetic commercial specimen.
Glutamic acid. Commercial specimen containing a mixture of the enantiomorphs.
Other compounds employed were all Analar reagents.
Indophenol titration. Solutions containing ascorbic acid or reductic acid were titrated directly with mN-2:6-dichlorophenolindophenol. With reductone or alkali-treated glucose solutions, however, direct titration was unsatisfactory owing to the slowness of the reaction. In these cases the reducing solution was run into an excess of acidified indophenol solution, allowed to react for 1 min., and the excess of the indicator back-titrated with stabilized mN-ascorbic acid solution.
Treatment of glucose solutions with varying concentrations of sodium hydroxide. 20 ml. 2·5% w/v glucose solution together with sufficient water to give a total volume of 25 ml. after the addition of the necessary quantity of NaOH were placed in a boiling tube in a water-bath at 91·5° so that the temperature inside the tube was 90°. After the addition of NaOH the solutions were mixed for a short time by a current of nitrogen; continuous bubbling of the gas was undesirable owing to evaporation. At the end of the required period the reaction was stopped by the addition of a small excess of HCl. The solutions were cooled in an atmosphere of nitrogen, diluted to a known volume, and the reducing value assessed by indophenol titration.

Buffers

For adjustment to ...	pH 3·5	pH 1·5
ml. 10% CCl_3CO_2H	417	417
g. NaCl	167	167
g. KH_2PO_4	23·3	23·3
g. citric acid	36	36
ml. 4 M-sodium acetate	123	30·4
Water	To 1 l.	To 1 l.

For convenience these solutions are referred to below as 'chloride-citrate buffers'.

For experiments at pH 2·0: 150 ml. 8% w/v metaphosphoric acid solution, 31·8 ml. M-secondary sodium citrate solution, water to 200 ml.

Reaction of reducing compounds with formaldehyde at different pH *values.* The reaction mixtures were made up as follows:

	pH 3·5	pH 1·5	pH 2·0
ml. stock buffer solution	20	20	20
ml. aqueous solution of added substances	20	24	20
ml. 12M-formaldehyde solution	2	2	10
Concentration of HCHO in the reaction mixture (M)	0·57	0·52	2·4

The pH of the reaction mixture was always checked by means of a glass electrode and adjustments made if necessary by the addition of HCl or sodium acetate solution.

The solutions containing approximately 0·9 mN reducing substance were allowed to attain the temperature of the thermostat (usually 25°). The HCHO solution was added at a given time; portions were subsequently withdrawn at intervals, acidified to stop the reaction and titrated with indophenol. Pure nitrogen was passed through the solutions during the whole experiment to prevent atmospheric oxidation. The initial titration value of the reducing compound was determined on a separate solution in which the HCHO was replaced by water.

Oxidation and reduction of alkali-treated glucose solutions. A suitable volume of the reducing solution was added to 25 ml. citrate buffer (to give a pH of 3·5 after dilution) and diluted to approximately 40 ml. A few drops of starch solution were added and the solution then titrated with 0·1 N-iodine until a permanent blue colour appeared. The volume was made up to 50 ml., H_2S was passed immediately through the oxidized solutions for 5 min. and the vessel then sealed and allowed to stand for the stated time. At the end of this period nitrogen was passed through the solution to remove the excess of H_2S. The complete removal of H_2S was ensured by testing the issuing gas with dry lead acetate paper for 5 min. The reducing value of the solutions was then assessed by titration with mN-indophenol.

The action of acid upon pectin solutions. 10 ml. of the solutions containing 1% w/v citrous pectin and the required concentration of acid were introduced into pyrex tubes and sealed. The ampoules were then heated in a boiling water-bath or in an oil-bath maintained at 120° for the required time, rapidly cooled and a sample titrated with indophenol.

RESULTS

Reducing power of solutions prepared by the treatment of glucose with alkali under varying conditions

The only record in the literature, as far as we are aware, of a quantitative experiment upon the indophenol-reducing capacity of glucose solutions treated with alkali is that of Kertesz (1934), who employed only one concentration of alkali. To clarify our problem, a 2% w/v glucose solution was heated at 90° with different concentrations of NaOH for varying times. The results of these experiments are

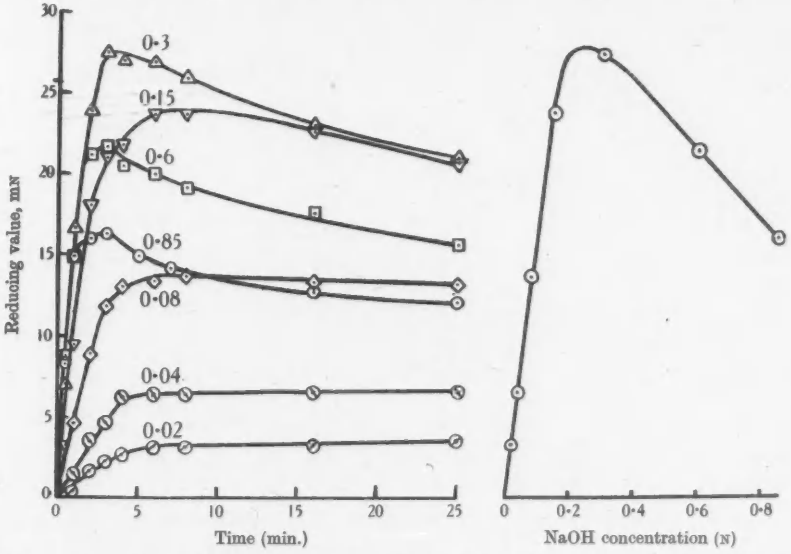


Fig. 1. The reducing value of solutions obtained by the treatment of glucose with varying concentrations of NaOH. Conditions of preparation: temp. 90°; glucose concentration 2% w/v. The figures against the curves indicate the concentration of NaOH (N). In the right-hand curve maximum reducing values are plotted against NaOH concentration.

represented graphically in Fig. 1, the reducing values being in terms of normality. It will be seen that at low concentrations of alkali the reducing value reached a maximum after about 6 min. and then remained almost constant. At higher NaOH concentrations the maximum was reached even earlier. In this case there was, in contrast to the lower concentrations, a slow diminution in the reducing value after the maximum had been reached. The maximum reducing value at low concentrations of alkali was directly proportional to the normality of the NaOH. With increasing concentrations, however, the maximum reducing power first increased more slowly and finally decreased. There was thus a particular combination of conditions under which the greatest possible concentration of reducing material could be obtained. This maximum occurred when the molar concentration of NaOH was about twice that of the glucose.

The action of formaldehyde on alkali-treated glucose solutions

The above experiments yielded data concerning glucose solutions heated for various times in the presence of various concentrations of alkali, and a range of such solutions differing from each other widely in the conditions of preparation were chosen for the investigation of their reaction with HCHO at pH 3·5. It would appear from Fig. 2 that the

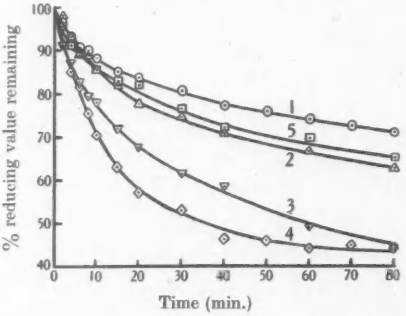


Fig. 2. The reaction of formaldehyde at pH 3·5 with alkali-treated glucose solutions prepared under different conditions. The figures against the curves refer to the alkali-treated glucose solutions as described in Table 2. Chloride-citrate buffer. Initial concentration of reducing substances approx. 0·9mN. HCHO concentration 0·57M. Temp. 25°.

conditions of preparation greatly influenced the course of the reaction with HCHO. The curve for solution 1 indicated a moderately rapid primary reaction succeeded by a much slower secondary reaction represented by an almost linear portion of the graph. At the other extreme the initial reaction with solution 4 was considerably more rapid. Although the rate of this reaction steadily declined there did not appear to be any evidence within the duration of the experiment of a succeeding linear reaction similar to that observed with solution 1. Curves for the other three solutions were intermediate in their characteristics. The striking fact is that none of the curves was similar in form to that obtained with purified reductone (see Fig. 3). Consequently the correction given for use with purified

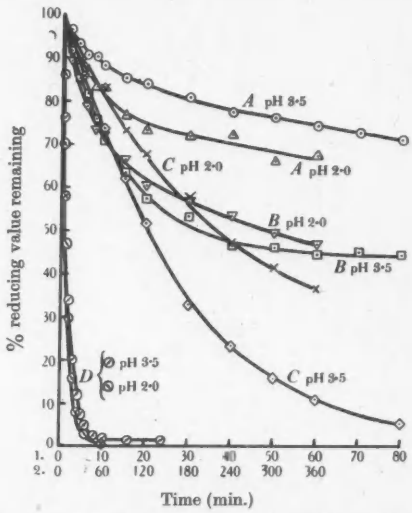


Fig. 3. Reaction of formaldehyde with reducing solutions at pH 3·5 and 2·0. *A*. Alkali-treated glucose solution 1 (see Table 2). *B*. Alkali-treated glucose solution 4 (see Table 2). *C*. Purified reductone. *D*. Ascorbic acid. Initial concentration of reducing solutions approx. 0·9mN. Temp. 25°. *pH* 3·5 *experiments*. Chloride-citrate buffer. HCHO concentration 0·57M. Time scale 1. *pH* 2·0 *experiments*. Metaphosphate-citrate buffer. HCHO concentration 2·4M. Time scale 2.

reductone (Snow & Zilva, 1943) cannot be applied successfully to alkali-treated glucose solutions because the course of the reaction is not linear in its initial stages. The failure of this type of correction applies also to the extrapolation recommended by Mapson (1943*a*, *b*).

The latter writer claims that, although the reaction at pH 3·5 is unsatisfactory, accurate results may be obtained by working at pH 2·0. We considered it necessary to ascertain the validity of this claim by comparing the behaviour of certain reducing solutions at pH 3·5 under our conditions with the behaviour of the same solutions at pH 2·0 under Mapson's conditions. The solutions chosen were ascorbic acid, purified reductone and alkali-

treated glucose solutions 1 and 4 (cf. Fig. 2), i.e. those showing the most extreme differences of behaviour. It was found that with ascorbic acid the velocity of reaction at pH 3·5 was almost exactly six times that at pH 2·0. In Fig. 3 the comparative results are given; the time scale for the pH 3·5 experiments is six times that for experiments at

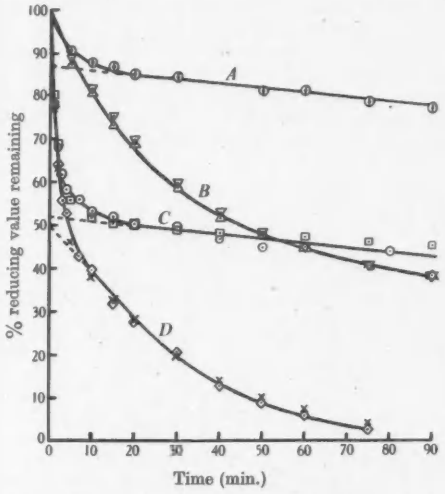


Fig. 4. The reaction of formaldehyde with mixed reducing substances at pH 3·5.

Curve	Reducing substances	Symbols: Observed values	Symbols: Values calculated by summation
A	Alkali-treated glucose solution (100)	ⓘ	—
B	Alkali-treated glucose solution (47) +reductone (53)	△	▽
C	Alkali-treated glucose solution (58) +ascorbic acid (42)	⊙	⊡
D	Reductone (49) +ascorbic acid (51)	◇	×

The figures in brackets indicate the percentage of the total initial reducing value contributed by each component. Corresponding curves for reductone and ascorbic acid alone are given in Fig. 3 to avoid repetition. Chloride-citrate buffer. Total initial concentration of reducing substances approx. 0·9 mN. HCHO concentration 0·57 M. Temp. 25°.

pH 2·0. It will be seen that the courses of the reactions at pH 2·0 were qualitatively very similar to those at pH 3·5. In the case of the purified reductone and solution 4 the velocity of the reaction compared with ascorbic acid was relatively slower at pH 2·0. With solution 1, however, at the lower pH the relative velocity was greater, and a greater fraction of the reducing material had combined at the point where the curve became linear. It is thus seen that these results do not uphold Mapson's assertion that the course of the reaction at pH 3·5 differs fundamentally from that at pH 2·0. Therefore no advantage is gained by carrying out the reaction at pH 2·0.

Another point of importance was to ascertain whether the course of the HCHO reaction with mixtures of different reducing compounds was equivalent to the sum of the reactions of the individual components. A typical experiment is illustrated in Fig. 4. In every case the observed results approximated very closely to the course of reaction calculated by summation of the reactions of the individual compounds. When the linear part of the curve (*A*) obtained with the alkali-treated glucose solution was extrapolated to zero time, the figure obtained corresponded to 13 % of the initial reducing value. In a mixture of the same reducing solution with a known quantity of ascorbic acid, approximately the same percentage of the reducing value remained after extrapolation. Thus in the case of curve *C*, in which 42 % of the total reducing value was due to pure ascorbic acid, extrapolation gave an 'ascorbic acid value' of 51·5 %. When curve *D* was extrapolated, the 'ascorbic acid value' of the solution obtained was 50 % of the total reducing value, a figure which agreed well with the proportion of ascorbic acid actually present (51 %). This result would be expected with purified reductone where the course of the reaction is linear in its initial stages. It may therefore be stated that when purified reductone is present, the error in Lugg's method is

Table 1. *Errors in the estimation of ascorbic acid by Lugg's method in the presence of reductone or of alkali-treated glucose solutions (calculated from Fig. 3)*

Reducing solution	pH of HCHO reaction	% of total reducing value obtained as 'ascorbic acid': Lugg's technique uncorrected	Corrected by formula	Corrected by extrapolation
Purified reductone	3·5	30	5	—
Purified reductone	2·0	22	7	—
Alkali-treated glucose solution 1	3·5	13	8	15
Alkali-treated glucose solution 1	2·0	20	15	22
Alkali-treated glucose solution 4	3·5	31	17	—*
Alkali-treated glucose solution 4	2·0	31	22	33

* No extrapolation was possible in this case because the curve did not become linear.

small whether corrected by extrapolation or by the application of our formula (vide Snow & Zilva, 1943). In the case of alkali-treated glucose solutions, however, a considerable error may be expected, the magnitude of which will vary according to the method of preparation of the reducing solution and which is thus unpredictable. An indication of the order of the error that may occur is given by the figures in Table 1. These results show the danger of the random application of Lugg's method, even when corrections are employed.

The differential behaviour of purified reductone and of alkali-treated glucose solutions after oxidation, and reduction with H_2S

In our previous paper we drew attention to the fact that when solutions of purified reductone are oxidized and subsequently reduced with H_2S at pH 3·5 only a small fraction of the original reducing value is regenerated. Mapson (1943*b*) found, on the other hand, that by reducing alkali-treated glucose solutions oxidized with iodine with H_2S for 5 hr., the entire reducing capacity could be regenerated. This suggested a further difference between the chemical behaviour of purified reductone and that of alkali-treated glucose solutions. We have therefore investigated the regeneration by H_2S reduction, after oxidation with iodine, of the reducing capacity of the alkali-treated glucose solutions used in the experiments of the preceding section. The results which are given in Table 2 show considerable differences between the various solutions. With solution 1

Table 2. *Recovery of reducing capacity from oxidized alkali-treated glucose solutions after treatment with* H_2S

Solution no.	Conditions of preparation: NaOH concentration (N)	Conditions of preparation: Time of heating (min.)	% of original reducing capacity recovered after oxidation and subsequent treatment with H_2S for (hr.): 1	5	20
1	0·02	10	93	100	107
2	0·15	5	76	83	87
3	0·3	5	71	75	83
4	0·3	0·5	55	62	75
5	0·85	15	82	85	93

the reduction with H_2S was very rapid and was complete at the end of 5 hr.; longer periods tended to lead to a reducing value in excess of that originally present. At the other extreme, solution 4 even after 20 hr. reduction showed only a 75 % recovery. With purified reductone it has been found that the recovery was extremely variable, but in general the figures were low and varied from 5 to 15 %. When Table 2 and Fig. 2 are examined it will be seen that the alkali-treated glucose solutions may be arranged in a series showing progressive changes of behaviour in both types of reaction. The order of the solutions in the series is the same in both cases.

The reducing power of solutions prepared by the treatment of pectin with acid under varying conditions

On theoretical grounds it would be expected that under most conditions prevailing in the preparation of food, reductic acid is more likely to be formed than reductone. It was therefore decided to investigate in detail the conditions under which reductic acid is formed from citrous pectin, a precursor of reductic acid. The pectin was heated for various periods of time in the presence of different concentrations of acid.

From the results given in Fig. 5 it may be assumed that little reductic acid would be formed during the preparation of foods at a temperature of 100°, since

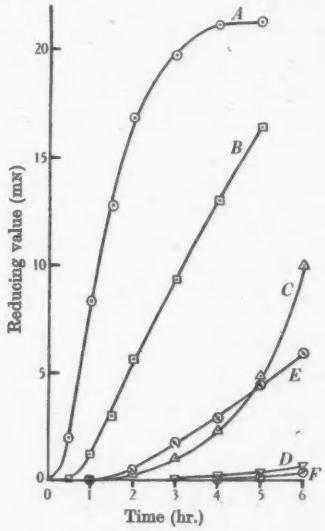


Fig. 5. The production of reductic acid from pectin by the action of acid under varying conditions.

Concentration of citrous pectin = 1 % w/v.

Temp.	Acid	Curve
120°	2N-H_2SO_4	*A*
120°	0·5N-H_2SO_4	*B*
120°	0·25M-citric acid	*C*
120°	No addition	*D*
100°	2N-H_2SO_4	*E*
100°	0·5N-H_2SO_4	*F*
100°	0·25M-citric acid	No detectable reducing value was obtained after 6 hr.
100°	No addition	

in the absence of acid or in the presence of 0·25M-citric acid no reducing substances were formed after 6 hr. Even in the presence of 0·5N-H_2SO_4 the

Table 3. *The reaction between formaldehyde and reductic acid or acid-treated pectin solution at* pH 3·5

Chloride-citrate buffer. Initial concentration of reducing substances approx. 0·9 mM. Concentration of HCHO 0·57 M. Temp. 25°.

Reducing solution	Percentage of initial reducing value remaining after (min.)										
	½	1	2	5	10	15	30	45	60	75	90
Reductic acid	95·4	94·6	94·6	92·6	91·8	90·4	88·4	87·6	86·3	86·0	85·5
Acid-treated pectin solution	96·3	96·3	95·5	94·2	91·9	90·8	89·2	87·1	86·9	86·4	86·4

amount was negligible until after the first 3 hr. On the other hand, with higher temperatures such as sometimes prevail in the processing and preparation of foods, indophenol-reducing substances are formed in quantities which may seriously interfere with the determination of ascorbic acid by titration with this indicator. It is to be noted that the production of reductic acid from pectin is not accompanied by the formation of brown substances such as are produced during the action of alkalis on sugar solutions.

The results given in Table 3 show that the acid-treated pectin solution reacted with HCHO at pH 3·5 in a similar way to reductic acid, and consequently it is very probable that this reducing compound only was formed during the treatment. It may be noted here that the course of the reaction between reductic acid and HCHO was somewhat different in the buffer solution employed in the present series of experiments from that used in the experiments described in the previous paper.

Accelerating influence of some substances on the rate of disappearance of ascorbic acid in the presence of formaldehyde at pH 1·5

It was shown in our previous paper that in certain extracts the rate of disappearance of ascorbic acid in the presence of HCHO at pH 1·5 was considerably increased, thereby introducing an error into the determination. The only compound which was then found to cause an acceleration under these conditions was glycine. Further experiments have now shown that other compounds are capable of producing the same effect. Amongst these the outstanding example is methyleneaminoacetonitrile, which is far more active in this respect than glycine. Fig. 6 shows that a concentration as low as 0·5 mM doubled the initial velocity of the disappearance of ascorbic acid at pH 1·5, whilst at a concentration of 40 mM more than 90 % of the ascorbic acid present had disappeared in 4 min. at 25°. The compound alone in the absence of HCHO did not react with ascorbic acid. It is unlikely that methyleneaminoacetonitrile occurs in any natural product, but the possibility of the occurrence of other compounds of similar activity must be borne in mind.

In our first paper we mentioned that some amino-acids did not have the accelerating effect of glycine. Further evidence is now produced that the behaviour of other amino-acids differs from glycine quantitatively rather than qualitatively. Thus it has been found that neither glutamic acid nor alanine had a marked effect at a concentration of

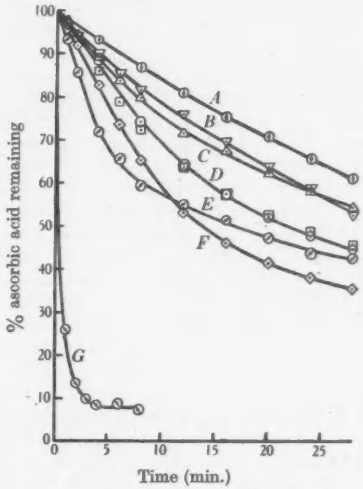


Fig. 6. The rate of disappearance of ascorbic acid at pH 1·5 in the presence of HCHO and various other substances.

Curve	
A	HCHO alone
B	50 mM methylamine
C	0·5 mM methyleneaminoacetonitrile
D	⊖ 10 mM glycine
	⊡ 10 mM ethyl aminoacetate
E	50 mM glutamic acid
F	50 mM alanine
G	40 mM methyleneaminoacetonitrile

In cases *A* and *C–G*, HCHO added at 0 min. In case *B* methylamine allowed to react with HCHO for 30 min. Ascorbic acid then added at zero time. Chloride-citrate buffer. Initial concentration of ascorbic acid 0·46 mM. HCHO concentration 0·52 M. Temp. 25°.

10 mM. When the concentration of these compounds was 50 mM, however, the initial velocity of combination of ascorbic acid was greatly increased (Fig. 6). It is of interest to note that esterification of glycine did not alter its action on the rate of disappearance of ascorbic acid (Fig. 6). A very slight accelerating

effect was observed with methylamine, but only at the high concentration of 50mM, and when the HCHO was allowed to react with the amine before the addition of ascorbic acid.

Fortunately, since in the above reactions the course of the disappearance of ascorbic acid was found to be practically linear in its initial stages, it is possible to introduce a correction when acceleration is suspected at pH 1·5. This will be dealt with in a later section.

DISCUSSION

The results of this investigation leave no doubt that by heating glucose in the presence of alkali two or more indophenol-reducing substances, of which reductone may be one, are produced. The expression 'reductone' is applied here to the compound isolated by Euler & Martius (1933). This compound, purified by sublimation, was found by us to behave like the unsublimed product. The production of several indophenol-reducing substances is in accord with Euler & Klussman's (1933) suggestion that at least three 'reductones' are formed on the treatment of a hexose with alkali. Mapson also considered that two substances are formed because of the behaviour on H_2S treatment after oxidation and because the reaction with HCHO appeared to occur in two stages. The latter argument in itself, however, does not prove the dual character of the reducing substance since single pure compounds such as reductic acid or 6-substituted ascorbic acids behave in a similar manner. It is highly probable that the reducing substances formed from glucose in Euler & Martius's method of preparation consist mainly of reductone since most of the reducing value of the solution after alkali treatment could be accounted for by us in the solid compound isolated according to their instructions. Euler & Martius heated their glucose and alkali solutions in the presence of lead acetate. It is known that the action of $Pb(OH)_2$ on glucose gives rise to breakdown products different from those produced by NaOH (Löb, 1909; Löb & Pulvermacher, 1910), and as no lead salt was present in the preparation of our alkali-treated solutions it is not surprising that they exhibited properties different from reductone. In our preparations which showed progressive changes in behaviour, the properties of solution 4 (Fig. 2 and Table 2) in regard to its reaction with HCHO and reduction with H_2S after oxidation suggested the presence of reductone amongst other reducing substances.

The point which is of particular interest to us is that the reducing substances in our alkali-treated solutions behaved differently from pure reductone. The possibility of the formation of reducing substances from hexoses under conditions usually prevailing in the preparation of food is not great because the medium is not sufficiently alkaline. When, however, higher temperatures are employed, such as are used in certain manufacturing processes, the likelihood of their formation is increased. It is of interest to note that heat and alkali, which conduce to the production of reducing substances, have at the same time a deleterious effect on ascorbic acid especially in the presence of air and consequently it would be very misleading if the resulting reducing value were taken as an index of ascorbic acid. The probability of the formation of reductic acid from pectin or pentoses, as already mentioned, might constitute a greater error in the determination of ascorbic acid in unknown solutions than the products produced from hexoses by alkali treatment. It is of importance to point out in this connexion, that whilst the production of reducing substances by the alkali treatment of glucose is accompanied by caramelization, this is not the case when reductic acid is produced. It would therefore be dangerous to assume that in the absence of caramelization no reducing substances capable of simulating the presence of ascorbic acid have been formed.

In our previous paper formulae for the correction of the results were given for solutions which contained pure reductic acid and reductone. The present investigation shows that the reductic acid formula is not applicable to unknown solutions since, if applied to reducing substances such as are found in alkali-treated glucose solutions, the error may even be increased. If, on the other hand, the reductone correction is applied, the error is definitely reduced although it may not be abolished.

In spite of the above shortcomings Lugg's formalin method still remains more specific than direct indophenol titration and if used with great caution may prove of use particularly in problems of a fundamental nature. Utilizing the knowledge so far available we have therefore worked out a procedure which to our minds yields the most satisfactory results. This procedure, which may in the first instance appear rather complicated, is really simple when put to the test.

MODIFIED PROCEDURE

Theoretical

It was our aim to simplify the method of the pH adjustments without sacrificing accuracy. In the preparation of the trichloroacetic acid extracts NaCl is added so that the pH of the acid solution is lowered and when the pH is eventually adjusted to 1·5 trichloroacetic acid is present mainly as the sodium salt. The addition of NaCl has the following advantages. The concentration of the trichloroacetic acid in the extract may under these conditions vary without greatly influencing the procedure of the pH adjustment. The high ionic concentration will eliminate any differences that may arise from the presence of inorganic constituents in the extracted material and thus avoid

changes in the velocity of the reaction which affect the accuracy of the method. Finally the NaCl will inhibit the catalytic oxidation of ascorbic acid in the presence of salts of heavy metals.

Another factor which must be considered in the adjustment of pH is the very marked influence of dilution upon pH in the 1·5 range. In the procedure described below the solution is initially adjusted to pH 1·39. This value was chosen because it has been found that, after dilution and treatment with HCHO under the conditions described, the final pH rises to exactly 1·50. At pH 3·5, on the other hand, the dilution and addition of HCHO does not alter the pH.

The adjustment of pH is carried out by addition of HCl or sodium acetate solution. Sodium acetate is used in place of alkali in order to avoid the destruction of ascorbic acid or dehydroascorbic acid by local alkalinity which might occur if NaOH were used. Over the range of the adjustment sodium acetate has a negligible buffering action and the solution is prepared so as to be equivalent in concentration to the acid used.

In order to adjust to pH 3·50 the necessary quantity of standardized sodium acetate is added to the extract which has already been brought to pH 1·39. The solution at pH 3·50 can then be adjusted to pH 1·50 by the addition of HCl equivalent to the sodium acetate added, provided that the correct conditions of dilution are observed. Thus all the data necessary for final adjustments are obtained in one operation.

As the composition of the solutions after adjustment by this procedure differs from that of the trichloroacetate-citrate buffers used in our previous investigation, we have redetermined the velocity constants of the combination of ascorbic acid with HCHO. The buffer solutions used for this purpose were also employed for all the other reaction velocity experiments in this paper, and their composition is described in the experimental section. The velocity constants are given in Table 4 together with the reaction times at different temperatures for use in the modified procedure. Since the pH adjustment involves dilution of the extracts, the volumes of the solutions have been chosen so as to simplify the calculation, and the factor for dilution is 1·2 throughout.

Table 4. *Constants for the reaction of ascorbic acid with formaldehyde at* pH 1·5 *and* pH 3·5

Velocity constant

$k = (k_1[F] + k_2)10^3$. (For symbols and units see Snow & Zilva, 1943.)

x_e = percentage ascorbic acid combined at equilibrium.

pH 1·5: the times given are those required to produce a loss of 1% ascorbic acid.

pH 3·5: the times given are those required to reach 99·5% equilibrium value.

Chloride-citrate buffers. HCHO conc.: pH 1·5, 0·52M; pH 3·5, 0·57M.

	pH 1·5		pH 3·5		
Temp.	k	Time (sec.)	k	Time (min.)	x_e
30°	28·2	22	890	6	98·2
25°	17·5	35	554	10	98·5
20°	10·2	60	311	17	98·9
15°	5·86	105	191	28	99·1
10°	3·49	175	104	51	99·3

The reactions with HCHO are carried out in a manner similar to that recommended by Lugg. After pH adjustment, three equivalent portions of the solution are treated as follows:

(1) Titrated at pH 1·5 with indophenol. (Titre $= v'$.)

(2) Allowed to react with HCHO at pH 1·5 for the standard time (calculated to cause the combination of 1% of ascorbic acid present) and titrated. (Titre $= v_1$.)

(3) Allowed to react with HCHO at pH 3·5 for the standard time (calculated to allow the reaction with ascorbic acid to reach 99·5% of the equilibrium value), and then titrated at pH 1·5. (Titre $= v_2$.)

Two different types of error may enter into the above determination, i.e. (*a*) due to the presence of substances which accelerate the reaction between HCHO and ascorbic acid at pH 1·5; (*b*) due to the presence of substances other than ascorbic acid which react with HCHO at an appreciable rate at pH 3·5. The results are therefore examined and corrections if necessary are made as follows:

(1) When $v' - v_1 < (v_1 - v_2)/25$ and $v_2 < v_1/6$, i.e. when the errors are so small that no further corrections need be applied.

These arbitrary limits were chosen for practical purposes so that the maximum error that can arise at pH 1·5 is 3%. The limit chosen for the pH 3·5 reaction is such that in a mixture of ascorbic acid and purified reductone the maximum error in the determination of ascorbic acid would be 5%.

Then if

v = titration of ascorbic acid present in a volume of the original extract equal to that used for the titrations,

x_e = percentage ascorbic acid combined at equilibrium after HCHO reaction at pH 3·5 (Table 4),

m = titration equivalent of substances of Class 1 (Lugg's classification),

n = titration equivalent of substances of Class 3 (Lugg's classification),

$$1{\cdot}2v' = v + m + n, \tag{1}$$

$$1{\cdot}2v_1 = \frac{99}{100}v + n, \tag{2}$$

$$1{\cdot}2v_2 = \frac{100 - x_e}{100}v + n. \tag{3}$$

Whence from (1) and (2) $v = \frac{120}{x_e - 1}(v_1 - v_2)$, the final formula used in the calculation.

(2) When $v' - v_1 > (v_1 - v_2)/25$ and $v_2 < v_1/6$. This may be due to interference by substances of Class 1 of Lugg's classification or to substances accelerating the disappearance of ascorbic acid in the presence of HCHO at pH 1·5. A further titration is performed after reaction with HCHO at pH 1·5 for twice the standard time. (Titre $= v_3$.) Then by a deduction similar to case 4 the results are calculated as follows:

$$v = \frac{120}{x_e}(2v_1 - v_2 - v_3).$$

(3) When $v' - v_1 < (v_1 - v_2)/25$ and $v_2 > v_1/6$. This indicates the presence of a considerable quantity of substances of Class 3 of Lugg's classification which may react to some extent with HCHO at pH 3·5. A further titration is carried out after reaction with HCHO at pH 3·5 for twice the standard time. (Titre $= v_4$.) Then, by a calculation similar to that given in case 4,

$$v = \frac{120}{x_e - 1}(v_1 - 2v_2 + v_4).$$

(4) When $v' - v_1 > (v_1 - v_2)/25$ and $v_2 > v_1/6$. In this case there is the possibility of interference from the sources indicated in both cases 2 and 3. Both additional titrations must then be performed and the required formula for the calculation is derived as follows:

Let r = fraction of ascorbic acid combined with HCHO at pH 1·5 in the standard time,

s = fraction of Class 3 compounds combined with HCHO at pH 3·5 in the standard time.

Assuming that the course of the reaction of ascorbic acid with HCHO at pH 1·5 in the presence of interfering substances and the reaction of Class 3 substances with HCHO at pH 3·5 are both linear, then

$$1{\cdot}2v' = v + m + n, \quad (1)$$

$$1{\cdot}2v_1 = v(1-r) + n, \quad (4)$$

$$1{\cdot}2v_3 = v(1-2r) + n, \quad (5)$$

$$1{\cdot}2v_2 = \frac{100 - x_e}{100} v + n(1-s), \quad (6)$$

$$1{\cdot}2v_4 = \frac{100 - x_e}{100} v + n(1-2s). \quad (7)$$

Whence from equations (4), (5), (6) and (7) the final formula $v = \frac{120}{x_e}(2v_1 - 2v_2 - v_3 + v_4)$ is obtained.

Practical

Solutions required. 4N-hydrochloric acid (standardized). 2N-hydrochloric acid (standardized).

Phosphate-citrate buffer made up as follows:

Citric acid	48 g.
Potassium dihydrogen phosphate	31 g.
Water	To 100 ml.

Standardized sodium acetate (approx. 4M). This solution is standardized by the following empirical procedure: 10 ml. of a solution containing approx. 57% w/v hydrated sodium acetate is titrated without dilution with 2N-HCl, with thymol blue as indicator. The end-point is taken as the first definite colour change in the acid range (yellow orange). The titration should be 20·6 ml. and the strength of the solution should be adjusted to give this value.

Preparation of extracts. Trichloroacetic acid is used for the purpose of extraction and the extracts must also contain approximately 10% w/v sodium chloride. For products containing about 80% moisture the following extracting solution is convenient, 1 ml. solution being used for each gram of tissue:

CCl_3CO_2H	50 g.
NaCl	180 g.
Water	To 1 l.

pH *adjustment.* The adjustment of pH is conveniently carried out in a glass electrode assembly having an electrode chamber of capacity approximately 50 ml. Stirring is achieved by the passage of a current of nitrogen which also prevents the oxidation of ascorbic acid in the extract by atmospheric oxygen. 4M-sodium acetate or 4N-HCl are added to the solution from 2 ml. automatic burettes. After standardization of the electrode system, 33·3 ml. extract are delivered by a special pipette into the electrode cell and 1·5 ml. phosphate-citrate buffer added. Nitrogen is bubbled through the solution and sodium acetate added slowly until the pH is exactly 1·39. If the initial pH is greater than 1·39 it may be brought to this value by the addition of HCl, but if it is considerably higher there is an indication that the trichloroacetic acid used in the extraction was too dilute. The volume of sodium acetate or acid solution added in this preliminary adjustment need not be noted. The sodium acetate burette is then reset at zero and the extract titrated to exactly pH 3·50. If too much acetate is accidentally added the solution may be back-titrated with 4N-HCl. The volume of acetate required = u. The adjusted solution is then run into a 40 ml. graduated flask. The electrode chamber is washed out with water, and the solution and washings made up to volume and mixed. This solution can now be brought to pH 1·5 when required by the addition of $u/4$ ml. 2N-HCl/5 ml.

Three 5 ml. portions of the solution adjusted to pH 3·5 are withdrawn and treated as follows: (1) Adjusted to pH 1·5 and titrated with indophenol. (2) Adjusted to pH 1·5, allowed to react with 0·25 ml. 12M-HCHO for the time given in Table 4 according to the temperature and titrated. (3) 0·25 ml. 12M-HCHO is added and the reaction allowed to proceed at pH 3·5 for the necessary time (Table 4). The solution is then adjusted to pH 1·5 and titrated.

A further 20 ml. of the solution adjusted to pH 3·5 are treated by passing a current of H_2S for 5 min., allowed to stand for 20 hr. and the excess of H_2S removed by means of a stream of N_2. Three 5 ml. portions of the reduced solution are then treated as above.

The results are examined and the calculation carried out as described above; further titrations are carried out if corrections are necessary.

SUMMARY

1. The production by heat of indophenol-reducing substances from glucose in the presence of alkali and from pectin in the presence of acid has been investigated under various conditions.

2. A mixture of at least two reducing substances is formed from glucose in the presence of alkali. One of these may be identical with reductone isolated by Euler & Martius (1933). The mixture of substances formed in solution behaves quite differently from reductone on treatment with HCHO or oxidation and subsequent reduction with H_2S.

3. Erroneous results are obtained when Lugg's method is applied to alkali-treated glucose solutions, and the error cannot be avoided. It is pointed out that carrying out the reaction at pH 2·0 instead of pH 3·5 is less convenient and leads to no greater accuracy.

4. Reductic acid is probably the only indophenol-reducing compound formed by the action of acid on citrous pectin. This reaction is more likely to occur under conditions similar to those used in the treatment of foodstuffs than the formation of reducing substances resembling those obtained by treating glucose with alkali.

5. The production of reductic acid occurs without the formation of deep-brown products and the danger of using caramelization as an index of the formation of non-ascorbic acid reducing substances is stressed.

6. Methyleneaminoacetonitrile is much more active than glycine in accelerating the disappearance of free ascorbic acid in the presence of HCHO at pH 1·5. The possibility of errors in Lugg's method due to other substances behaving in a similar manner is emphasized.

7. A convenient procedure based on Lugg's formalin method is described.

One of us (G. A. S.) is indebted to the Medical Research Council for a whole-time grant. Thanks are also due to Roche Products Ltd. for a gift of *l*-ascorbic acid.

REFERENCES

Euler, H. v. & Klussman, E. (1933). *Ark. Kemi Min. Geol.* **11** B, no. 12.
—— & Martius, C. (1933). *Liebigs Ann.* **505**, 73.
Kertesz, Z. I. (1934). *J. biol. Chem.* **104**, 483.
Klages, A. (1903). *Ber. dtsch. chem. Ges.* **36**, 1506.
Löb, W. (1909). *Biochem. Z.* **20**, 516.
—— & Pulvermacher, G. (1910). *Biochem. Z.* **23**, 10.
Lugg, J. W. H. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 273.
Mapson, L. W. (1943*a*). *Nature, Lond.*, **152**, 13.
—— (1943*b*). *J. Soc. chem. Ind., Lond.*, **62**, 223.
Snow, G. A. & Zilva, S. S. (1938). *Biochem. J.* **32**, 1926.
—— —— (1941). *Biochem. J.* **35**, 787.
—— —— (1943). *Biochem. J.* **37**, 630.

The Micro-volumetric Determination of Iron, with Particular Reference to Blood

By W. N. M. RAMSAY, *Department of Physiology, Royal (Dick) Veterinary College, Edinburgh*

(*Received* 23 *August* 1944)

Modern research into the biochemistry of respiratory pigments demands iron determinations of an accuracy which is beyond that of the usual colorimetric procedures. Even such sensitive and specific reagents as $\alpha\alpha'$-dipyridyl and thiolacetic acid cannot surmount the difficulties inherent in the visual matching of colours. It is surprising, therefore, that in this field so few attempts have been made to apply to biological materials any of the volumetric methods of modern inorganic chemistry. Dubnoff & Kirk (1936) reduced iron to the ferrous state with cadmium-amalgam and titrated with standard $Ce(SO_4)_2$. They claimed to be able to determine as little as 2–15μg. Fe, but in this laboratory all reductions with pure metals and amalgams have led to 'blanks' of several μg., which impair greatly the accuracy of the results. Pincussen & Roman (1931) and McFarlane (1932) titrated ferric iron with standard titanous salts, but both methods require about 1 mg. Fe for each estimation. This precludes their use for many biological purposes.

This paper describes a titrimetric technique which can conveniently be used for the determination of any quantity of iron not less than 8–10μg.

METHOD

Principle of the method. Organic matter is destroyed by heating with H_2SO_4, $HClO_4$ and HNO_3 in a small pyrex flask (10–12 ml. capacity, internal neck diameter 8 mm., over-all length 80–90 mm., kindly blown for me by Mr C. Bell, Department of Biochemistry, University of Edinburgh). The digest is diluted and titrated with standard $Ti_2(SO_4)_3$, using a burette modified from the design of Conway (1939) so as to permit the storage of the $Ti_2(SO_4)_3$ under hydrogen (see Fig. 1). KCNS is used as indicator, and CO_2 for stirring.

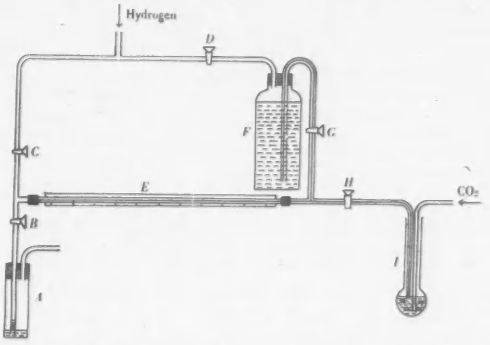


Fig. 1. Modified Conway burette. *A*, pressure-regulating vessel. *B*, *C*, *D*, *G* and *H*, stop-cocks. *E*, the burette, standard bore capillary tubing against mm. scale. 10 cm. $\simeq$ 0·05 ml. *F*, $Ti_2(SO_4)_3$ reservoir, capacity 200–250 ml. *I*, digestion and titration flask.

The method was originally designed for use with blood and solutions of haemoglobin pigments, but some of its limitations have been investigated with a view to its application to other biological material. It has been found that small amounts of copper cause the results to be irregular and high, but a modified technique has been worked out for use in the presence of Cu. This is based on the observation, believed to be original, that when a sulphuric acid solution of Fe^{+++} and Cu^{++} is saturated with NaCl or KCl and extracted with ether containing a little formic acid, the ferric iron alone passes into the ether layer.

Reagents. All reagents should be of A.R. quality unless otherwise stated.

Acids. H_2SO_4, HNO_3, $HClO_4$ (60%). The HNO_3 should be tested for iron, and distilled from glass if necessary.

Thiocyanate. KCNS, 50% solution in distilled water.

Titanous sulphate. $Ti_2(SO_4)_3$, N/50 to N/20 (depending on the quantity of iron and the bore of the burette). 200 ml. freshly boiled distilled water are poured into the bottle *F* (Fig. 1). The cocks *C*, *D* and *G* are closed, but *B* and *H* may be left open. An appropriate quantity of commercial 15% $Ti_2(SO_4)_3$ (or $TiCl_3$) is mixed with 10 ml. of water and 10 ml. conc. H_2SO_4, and the mixture is boiled briskly for a full minute. The solution is at once poured into *F*, which should be immediately stoppered. The cock *D* is opened, and the apparatus is left overnight, so that a precipitate which sometimes forms in the titanium solution may settle. Next morning *D* is closed, *C* is opened, and hydrogen is allowed to flow briskly from the generator. When the gas is bubbling freely through *A*, *C* is again closed, and first *D* and then *G* are opened. The pressure of hydrogen should be sufficient to drive the $Ti_2(SO_4)_3$ over the siphon into the burette. The lubrication of the cocks *G* and *H* is important, since most greases allow a slight but serious leakage of the strongly acid reagent. W. Edwards & Co. (London), Ltd., have kindly supplied me with a sample of their 'Soft Vacuum Grease', which has proved more satisfactory than other commercial lubricants. The reagent, even if the most stringent precautions are taken to exclude air, still oxidizes slowly, and must be standardized daily by titration against a standard iron solution.

Standard ferric iron. A very accurate standard solution may be prepared from a weighed quantity of pure iron wire, but for all ordinary purposes it has proved sufficient to dissolve in 500 ml. of approximately 3N-H_2SO_4 such a weight of $Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$ that 5 ml. of the solution require for reduction a volume of $Ti_2(SO_4)_3$ which alters the reading on the burette by 100–200 mm.

Reagents required only in the presence of copper

Sodium chloride. Solid, powdered.

Ether and *formic acid.* These need not be A.R., but must be redistilled from glass. They should be mixed only just before use.

Procedure

Digestion. This is described here for blood, but it has been shown by Levvy (1943), and confirmed in this laboratory, that the same reagents are satisfactory for the digestion of other tissues. 0·2 ml. of blood (a smaller volume may be used if great accuracy is not required) is measured into one of the flasks already described, followed by 0·2 ml. each of conc. HNO_3 and 60% $HClO_4$, and 0·5 ml. conc. H_2SO_4. A small glass bead is added, and the flask is gently heated, with continuous shaking, over a micro-burner. The HNO_3 boils off first, the mixture darkens, and clears again as the boiling-point of H_2SO_4 is approached. Just before the last of the $HClO_4$ boils off, the mixture turns a clear deep greenish-yellow colour for 3–5 sec. Heating is continued for 30 sec. after the disappearance of this colour. The cooled digest is diluted with 5 ml. of water (unless Cu is present; see below), and is then ready for titration. The digestion of 0·2 ml. of blood usually requires about 6 min. It is, of course, easy to destroy all the organic matter of blood with H_2SO_4 and HNO_3, but in the absence of $HClO_4$ some difficulty is experienced in getting rid of the last traces of HNO_3. Moreover, if H_2SO_4 and $HClO_4$ are used without HNO_3, it is much more difficult to prevent loss of material by bumping and splashing.

Separation of iron from copper (unnecessary in the analysis of blood). After the digestion the contents of the flask are diluted with 2 ml. of water and transferred as completely as possible to a narrow test-tube. The flask is rinsed with two successive portions of 1 ml. of water, each being transferred to the test-tube. 0·5 ml. of conc. H_2SO_4 is added, and the solution in the test-tube is saturated with NaCl. The tube is then shaken twice with 2 ml., and twice with 1 ml. of ether containing 5% (by vol.) of formic acid. The ether extracts are transferred back to the original flask, and the ether is evaporated over a warm water-bath. The formic acid has a tendency to form non-volatile polymers, so it is necessary to repeat the digestion, after the addition of 0·5 ml. of conc. H_2SO_4 and a drop or two each of HNO_3 and $HClO_4$. Care must be taken to remove all the ether before the concentrated acids are added, or a minor explosion may result in the loss of most of the material. The digest is finally cooled and diluted with 5 ml. of water.

Titration. The titration technique is simple, but accurate results depend on attention to certain details. After the burette has been flushed out five or six times and the $Ti_2(SO_4)_3$ standardized (see below), the diluted digest, plus 1 ml. of 50% KCNS, is so placed that the tip of the burette dips just below the surface of the solution. The tube which delivers the stream of CO_2 should be 3–5 mm. longer. If it is lashed to the tip of the burette, the two fine tubes reinforce each other and are less liable to break. CO_2 is bubbled through the flask, the cock *H* is turned on, and $Ti_2(SO_4)_3$ is allowed to run out of the burette until the last trace of pink colour just disappears. The reaction between Fe^{+++} and Ti^{+++} is not instantaneous, even in the presence of a catalyst such as KCNS, and it is therefore necessary to regulate carefully the rate of flow from the burette. This is done, as with the simple Conway burette, by adjusting the height of the column of fluid in the tube leading from *A* to *B*, and the titration is finally done with the cock *C* closed and *B* open. The fluid should leave the burette at a rate of 0·01–0·02 ml./min. The last few μl. may be run out even more slowly if *B* is closed just before the end of the titration. The lighting used for the observation of the end-point is particularly important. The apparatus should be placed in a dull corner, a white card pinned behind the flask, and light from a 'daylight' blue bulb thrown on it from a few inches away. An aluminium reflector increases the illumination and shields the eyes of the operator. Under these conditions a perceptible change in colour (towards the end of a titration) is produced by the addition of 0·0005–0·001 ml. N/25 $Ti_2(SO_4)_3$.

Standardization is carried out by the titration of 5 ml. portions of ferric ammonium sulphate solution in exactly the same way.

RESULTS

The analysis of fourteen 0·2 ml. samples of the same blood, using the simple direct procedure, gave concentrations of 51·66, 52·05, 52·24, 52·10, 51·97, 51·93, 51·57, 51·75, 51·97, 51·36, 51·92, 51·95, 52·36, 52·00 mg. Fe/100 ml. blood. The standard deviation of a single observation from the mean was 0·25 mg./100 ml. This corresponds to a coefficient of variation of 0·48 %, which agrees well with the value of 0·45 % calculated from a large series of observations made in triplicate and quadruplicate. The error of the procedure modified for Cu is somewhat greater, for two reasons: the additional manipulation is contributory, and also consumes time, but more serious is the fact that all samples of NaCl hitherto examined contain significant traces of iron. This leads to blank titrations much higher and more variable than those encountered in the simple method.

Table 1 gives the results of some experiments on the recovery of known amounts of Fe added (as ferric ammonium sulphate) to 0·1 or 0·2 ml. samples of blood before digestion. Table 2 illustrates the separation of Fe from Cu by extraction with ether-formic acid.

Table 1. *Recovery of iron added to blood*

Fe added (μg.)	Fe recovered (μg.)	Recovery (%)	Fe added (μg.)	Fe recovered (μg.)	Recovery (%)
10·1	9·7	96·0	30·0	29·3	97·7
10·1	10·6	105·0	47·0	47·6	101·3
30·0	29·9	99·7	59·0	59·6	101·0
30·0	29·6	98·7	83·0	83·0	100·0
30·0	30·4	101·3	94·0	95·3	101·4
30·0	29·5	98·3	106·0	104·8	98·8
30·0	29·5	98·3	118·0	118·0	100·0

Table 2. *Separation of iron from copper by extraction with ether-formic acid mixture*

Material analyzed	Fe found (μg.)
0·2 ml. blood	101·6, 100·8
0·2 ml. blood + 15·3 μg. Cu (not extracted)	119·6, 111·5
0·2 ml. blood + 15·3 μg. Cu (extracted)	100·9, 100·4

DISCUSSION

The results quoted show that in the absence of copper the method is capable of giving highly accurate results. It has been in use in this laboratory for some time, and has given much more consistent results than αα′-dipyridyl or thiolacetic acid. In theory, it is lacking in specificity, since titanous salts react with a variety of oxidizing agents. The digestion, however, destroys all organic compounds, and in most biological material the ratio of iron to other inorganic redox systems is so high that the latter may, with certain reservations, be ignored. In the case of blood no difficulty arises, since the only other redox system surviving the digestion is copper, and the ratio of iron to copper is of the order of 300–400 to 1; but in other tissues, notably liver, the ratio may be 20 to 1 or even lower, with the result that the quantity of Ti^{+++} used in the titration may be considerably in excess of that required for the Fe present.

The observation that ferric iron in the presence of NaCl and H_2SO_4 is soluble in a mixture of ether and formic acid is obviously related to the fact that when $FeCl_3$ in exactly 6N-HCl is shaken with ether, the iron passes into the ether layer (Szaffka, 1929; cf. Vogel, 1939). The explanation in both cases lies no doubt in the formation of ether-soluble complexes. The presence of excess Cl^- should depress the ionization of $FeCl_3$ and facilitate the formation of the double molecule Fe_2Cl_6. This can be regarded as the ferric salt of the hypothetical acid H_3FeCl_6, analogous to $H_3Fe(CN)_6$ and $H_3Fe(CNS)_6$. The fact that the chlorides of Au^{+++}, Mo^{+++} and Ti^{+++}, which form complexes very readily, are also extracted into ether from 6N-HCl, tends to support this theory. The concentration of HCl in the experiments of Szaffka was critical, but in the experiments described here a good deal of latitude is permissible. Even with N-H_2SO_4 there is a perceptible passage of iron from the aqueous to the ethereal layer. The effect becomes pronounced at an acid concentration of about 3N, and approaches its maximum at about 5N. Increasing the concentration of acid, at least up to 9N, exercises no adverse influence. For practical purposes, also, the proportion of formic acid in the ether makes no difference. Equally efficient extractions have been obtained with ether containing 5, 10 and 20 % of formic acid.

SUMMARY

1. A titanometric method is described for the determination of 10–200 μg. iron.

2. The coefficient of variation, when the method is applied to 0·2 ml. portions of blood, is 0·45 %.

3. Copper interferes, but may be separated from iron by saturating the solution with NaCl in dilute H_2SO_4, and shaking with ether containing formic acid, when the iron passes into the ether layer.

The author is indebted to Prof. Henry Dryerre for encouragement and advice.

REFERENCES

Conway, E. J. (1939). *Microdiffusion Analysis and Volumetric Error*. London: Crosby Lockwood.
Dubnoff, J. & Kirk, P. L. (1936). *Mikrochemie*, **19**, 194.
Levvy, G. A. (1943). *Biochem. J.* **37**, 598.
McFarlane, W. D. (1932). *Biochem. J.* **26**, 1034.
Pincussen, L. & Roman, W. (1931). *Biochem. Z.* **231**, 54.
Szaffka, T. (1929). *Mag. chem. Foly.* **35**, 44. Quoted in *Brit. chem. Abstr.* A (1931), p. 188.
Vogel, A. I. (1939). *Textbook of Quantitative Inorganic Analysis*. London: Longmans, Green & Co.

Ferrihaemoglobinaemia * (Methaemoglobinaemia) in Man and the Horse

By W. N. M. RAMSAY, *Department of Physiology, Royal (Dick) Veterinary College, Edinburgh*

(*Received* 26 *September* 1944)

Ferrihaemoglobin has been known for many years to occur in blood under a great variety of pathological and experimental conditions, but only recently has the suggestion been made that small amounts may also be present normally. The matter, however, is still a subject for controversy. Ammundsen (1941) has observed in 40% of 82 normal human blood specimens that the carbon monoxide capacity of the fresh blood was less than the CO capacity after reduction with $Na_2S_2O_4$, by amounts which varied up to 2·5 vol. %. The discrepancy, it is suggested, may be due to the presence of ferrihaemoglobin. These figures are supported by those of Havemann, Jung & Issekutz (1939), in whose hands spectrophotometric methods have shown that in healthy persons up to 8% of the total blood pigment may be present as ferrihaemoglobin. On the other hand, Cox & Wendel (1942), using a method (Wendel, 1938) which is claimed to detect ferrihaemoglobin spectroscopically when its concentration exceeds 4% of the total pigment, deny the normal existence of ferrihaemoglobin in the blood of man and numerous other species. In the case of man, this is understandable, since only in a few individuals have other workers found the proportion to be above 4%.

This paper comprises the results of a gasometric investigation of normal human and horse blood, with a view to the provision of further evidence on the point at issue. Ferrohaemoglobin has been determined, by means of its oxygen capacity, in all the specimens of blood. 'Total haemoglobin' (a phrase used here to indicate the sum of ferrohaemoglobin and ferrihaemoglobin, but excluding carbon monoxide haemoglobin) has been determined, again in all the specimens, by a technique which is also based on the oxygen capacity of ferrohaemoglobin. This procedure, developed by various workers from the original of Conant & Fieser (1924–5), is believed to be sounder than any involving the use of CO, since oxygen is a more specific reagent for ferrohaemoglobin than is CO. The latter combines not only with ferrohaemoglobin and a number of haemochromogens, but also with a series of compounds intermediate between these and the bile pigments (Lemberg, Legge & Lockwood, 1941). A significant difference between ferrohaemoglobin and 'total haemoglobin' may be regarded as being due to the presence of ferrihaemoglobin, especially as the technique for the determination of 'total haemoglobin' does not embrace carbon monoxide haemoglobin.

* In order to avoid confusion which might otherwise ensue, the terms ferrihaemoglobin and ferrohaemoglobin are used throughout this paper instead of methaemoglobin and haemoglobin (cf. Coryell, Stitt & Pauling, 1937).

METHODS

Collection of blood

Since ferrihaemoglobin can act as a hydrogen acceptor in certain enzymic oxidations (Warburg, Kubowitz & Christian, 1930 *a*, *b*), all blood specimens have been drawn into flasks surrounded with ice and containing powdered potassium oxalate as anti-coagulant. The rapid cooling should inhibit enzymic reduction of ferrihaemoglobin almost completely.

Analytical methods

Ferrohaemoglobin determinations. These have been made by the standard oxygen capacity technique (Van Slyke & Neill, 1924).

Iron determinations. These have been made either colorimetrically with thiolacetic acid and ammonia, or, more recently, by the more accurate titanous sulphate method of Ramsay (1944).

'*Total haemoglobin*'. This has been estimated by a modification of the procedure of Conant, Scott & Douglass (1928). It seems desirable to report the modified method in full, because it is somewhat simpler, and no less accurate than the original. The main advantages are that the determination can be carried out on a much smaller sample of blood, and entirely in the extraction chamber of the Van Slyke manometric apparatus. The principle remains unaltered: determination of oxygen capacity after reduction with a faintly alkaline titanium solution.

Reagents. (1) $TiCl_3$: commercial 15–20% $TiCl_3$ diluted with an equal volume of N-HCl, and stored in the usual way under hydrogen. It is convenient to include a burette in the permanent set-up.

(2) Alkaline citrate: 0·1 M-citric acid containing such a quantity of NaOH that, when 9 ml. are mixed with 1 ml. $TiCl_3$, the pH of the mixture lies between 7 and 8. The quantity of NaOH required varies according to the acidity of different commercial preparations of $TiCl_3$. Conant used a similar solution containing tartrate, borate and alkali, but the author prefers the citrate, which is easier to prepare and to use.

(3) 'Titanous citrate': a solution prepared not more than an hour or so before use (and preserved from air under

liquid paraffin) by diluting 1 ml. $TiCl_3$ to 10 ml. with the alkaline citrate solution. The pH of such mixtures should be checked occasionally, but it is worth noting that round about pH 8 'titanous citrate' solutions change colour from mauve to greenish black. Solutions of ferro- and ferrihaemoglobin, moreover, do not seem to be denatured by alkali unless the pH exceeds 10 (Conn, Kistiakowsky & Roberts, 1940).

(4) 0·5% $K_3Fe(CN)_6$ containing 0·1% octanol and either 0·3% white saponin or 0·25% Lissapol C. (a long-chain sulphonic acid detergent kindly supplied by Imperial Chemical Industries Ltd.).

(5) NaOH and $Na_2S_2O_4$ for the absorption of CO_2 and oxygen (Peters & Van Slyke, 1932).

Procedure. This description should be intelligible to anyone who is familiar with the manipulation of the Van Slyke manometric apparatus. 1 ml. blood or pigment solution is admitted to the extraction chamber from a rubber-tipped 1 ml. pipette, and followed by 1 drop octanol. The chamber is evacuated, and shaken gently for 3 min., in order to remove oxygen, which might otherwise interfere with the subsequent reduction. The vacuum is released, the liberated gases are expelled, and the extraction is repeated. If much ferrohaemoglobin is present, the colour of the solution at this point suggests that most of the oxygen has been removed. On the other hand, solutions containing a very high proportion of ferrihaemoglobin contain little oxygen, and the preliminary extraction may be omitted. Experiment has shown that small proportions of carbon monoxide haemoglobin remain unaffected by this extraction of gases.

A few ml. water are poured into the cup, and 0·25 ml. 'titanous citrate' is run into the chamber from a rubber-tipped, subdivided 1 ml. pipette. The stopcock is washed through by running in 0·25 ml. water, and the chamber is again evacuated. It is shaken gently for 5 min., during which period ferrihaemoglobin is reduced to ferrohaemoglobin. After the reduction the vacuum is partially released by the admission of mercury, and air is then allowed to fill the extraction chamber at atmospheric pressure. The apparatus is next shaken briskly for 5 min., to ensure the complete oxygenation of all ferrohaemoglobin. At the same time the excess titanium is oxidized to the inert titanic state, a process which occurs very rapidly in the presence of air. When oxygenation is complete, all gases are expelled from the chamber and the cock between the chamber and the cup is closed. 3 ml. $K_3Fe(CN)_6$ solution (previously freed from dissolved oxygen by extraction in the Van Slyke apparatus and stored, if necessary, under liquid paraffin for a few hours) are run into the cup, and 2 ml. are admitted to the extraction chamber. The cock is then sealed with mercury. The chamber now contains 3·5 ml. fluid, and the remainder of the analysis is precisely similar to the usual oxygen determination. When the calculation is made, however, there is a small difference. This arises from the fact that to each 1 ml. of pigment solution there has been added 0·25 ml. titanous citrate and 0·25 ml. water before equilibration with oxygen. This means that the 'c' correction must be larger than that used in the straightforward oxygen determination, because it must include oxygen dissolved physically in the additional 0·5 ml. solution. It is necessary to determine the 'c' correction accurately, using 0·25 ml. titanous citrate, 0·25 ml. water (these being equilibrated with air by mixing and shaking in the extraction chamber) and 3 ml. air-free $K_3Fe(CN)_6$. Variations in temperature should theoretically cause fluctuation of the correction, but in practice this is so slight as to be negligible.

The method has been tested on ferrihaemoglobin solutions prepared from crystallized horse oxyhaemoglobin by oxidation with nitrite or ferricyanide, followed by dialysis against distilled water. If concentrated solutions are required, precipitation must be prevented by dialysis against 0·1 M-Na_2HPO_4 or 0·1 M-$Na_2B_4O_7$. All dialyses have been done at about 2°. There is no apparent difference in behaviour between preparations made with ferricyanide and those made with nitrite. No independent accurate method of determining ferrihaemoglobin has been available, so that it has been rather difficult to check the accuracy of the results, but the oxygen capacity after reduction has been compared with the protein-iron content (iron precipitated by trichloroacetic acid).

RESULTS

All figures quoted here have, where necessary, been corrected for dissolved oxygen in accordance with the recommendations of Sendroy, Dillon & Van Slyke (1934).

Table 1 gives the results of the determination of total haemoglobin in 'pure' ferrihaemoglobin solutions, and a comparison of the oxygen capacities found with the protein-iron contents of the solutions. The analytical results show clearly that the procedure described is satisfactory for the determination of ferrihaemoglobin.

Table 1. *Analysis of 'pure' ferrihaemoglobin solutions*

Reagent*	Total haemoglobin (vol. %)	Fe (mg./100 ml.)	ml. O_2/55·84 mg. Fe
$K_3Fe(CN)_6$	4·48, 4·57, 4·62 4·52, 4·51, 4·61	11·4	22·3
$NaNO_2$	8·41, 8·51	21·7	21·8
$NaNO_2$	9·79, 9·74	24·0	22·7

* Reagent used for preparation of ferrihaemoglobin.

Ferrohaemoglobin and total haemoglobin have been determined in samples of blood from 38 normal human beings, all from males except no. 16, which was from a female. Table 2 gives the results of these analyses, with the differences between ferrohaemoglobin and total haemoglobin calculated as vol. O_2% and also as percentages of the total haemoglobin present. The accuracy of the analyses does not warrant the calculation of the percentages more closely than to the nearest 0·5%. In Table 3 are given the results of similar analyses made on 17 samples of blood from apparently normal horses. All determinations, except five marked with an asterisk in the tables, were made at least in duplicate, and some of the more surprising figures in Table 3 were confirmed by triplicate or even quadruplicate analysis.

Table 2. *Ferrohaemoglobin and total haemoglobin content of human blood*

Subject	Ferro-haemoglobin (vol. %)	Total haemoglobin (vol. %)	Difference Vol. %	Difference % of total haemo-globin
1	16·86	17·06	0·20	1
2	19·55	20·93	1·48	7
3	20·05	20·52	0·47	2·5
4	20·92	21·17	0·25	1
5	20·20	21·04	0·84	4
6	20·37	21·03	0·66	3
7	19·73	20·62*	0·89	4·5
8	19·40	19·96	0·56	3
9	20·49	20·37	−0·12	−0·5
10	20·81	21·39	0·58	2·5
11	19·62	20·25	0·63	3
12	21·37	21·62	0·35	1·5
13	20·74	21·55	0·81	3·5
14	19·68	19·68	0·00	0
15	19·89	20·06	0·17	1
16	15·42	15·94	0·52	3
17	19·99	20·28	0·29	1·5
18	19·78	19·85	0·07	0·5
19	21·03	21·06	0·03	0
20	20·11	20·45*	0·34	1·5
21	21·93	21·85	−0·08	−0·5
22	20·80	20·80*	0·00	0
23	20·53	20·56	0·03	0
24	20·17	20·33	0·16	1
25	21·10	21·13	0·03	0
26	20·40	20·93	0·53	2·5
27	20·62	21·33	0·71	3·5
28	20·26	20·22	−0·04	0
29	19·30	19·64	0·34	1·5
30	19·52	20·62	1·10	5
31	19·49	19·46	−0·03	0
32	21·85	22·07	0·22	1
33	19·13	19·70	0·57	3
34	20·07	21·32*	1·25	6
35	19·08	19·88	0·80	4
36	20·36	20·94	0·58	3
37	21·80	21·88	0·08	0·5
38	20·42	20·63	0·21	1

* These determinations were not made in duplicate.

Table 3. *Ferrohaemoglobin and total haemoglobin content of horse blood*

Horse	Ferro-haemoglobin (vol. %)	Total haemoglobin (vol. %)	Difference Vol. %	Difference % of total haemo-globin
1	14·34	14·80	0·46	3
2	14·73	19·82	5·09	25·5
3	19·76	20·36	0·60	3
4	17·56	21·35	3·79	17·5
5	17·00	18·54	1·54	8
6	14·81	15·55	0·74	4·5
7	14·76	15·90	1·14	7
8	16·94	17·12*	0·18	1
9	17·99	19·37	1·38	7
10	16·19	17·14	0·95	5·5
11	17·45	18·56	1·11	6
12	14·75	15·18	0·43	3
13	15·74	15·76	0·02	0
14	16·40	17·21	0·81	4·5
15	15·09	17·28	2·19	12·5
16	16·14	18·06	1·92	10·5
17	16·87	16·96	0·09	0·5

* This determination was not made in duplicate.

DISCUSSION

The standard error of a single oxygen determination, either before or after reduction with 'titanous citrate', is about 0·06 vol. %. It has therefore seemed statistically sound to conclude that differences between ferrohaemoglobin and total haemoglobin of not less than 1·5 %, or about 0·3 vol. %, may be considered real. It is believed that there are no chemical or manipulative grounds to invalidate this conclusion, and, in any case, many of the differences, particularly in the equine analyses, are so much greater than this limit that there can be no question of their reality. The few negative differences recorded in Table 2 are well within the limits of error, and may therefore safely be ignored.

If the above statistical standard be accepted, it then appears that in 21 human cases out of 38, and 14 equine cases out of 17, a significant difference has been found between ferrohaemoglobin and total haemoglobin. Bearing in mind the basis of the analytical methods employed, this is *prima facie* evidence for the presence, in certain cases, of ferrihaemoglobin, but conclusive proof is lacking. The figures are supported by the similar ones of Ammundsen (1941), which were obtained by a less specific method, and both sets bear out the contention of Havemann *et al.* (1939) that human blood may contain ferrihaemoglobin. There remain the contrary findings of Cox & Wendel (1942), which are difficult to reconcile with those of the other workers. It is true that in none of his samples has the present author been able to demonstrate the ferrihaemoglobin absorption band at about 630 mμ, but it is now generally admitted that spectroscopic examination for this band is far from being a sensitive test for ferrihaemoglobin in the presence of much oxyhaemoglobin (cf. Campbell & Morgan, 1939). Furthermore, it has not so far been possible in this laboratory to make the procedure of Wendel (1938) nearly as sensitive as that author claims.

One of the most striking observations that has been made in these experiments is that there is a marked quantitative difference between man and the horse. The percentage of horse bloods showing a significant difference between ferrohaemoglobin and total haemoglobin (about 80 %) is greater than that of human bloods (55 %). Moreover, the average difference in such samples between ferrohaemoglobin and total haemoglobin is very much greater (horse 1·58 vol. %, human 0·68 vol. %). Further work on the differences is therefore much easier in the case of horse bloods, and it is mainly as a result of additional work on that species that additional evidence (described below) has been accumulated.

Iron determinations have been made on many of the blood specimens analyzed, and in every case the amount of oxygen bound by 55·84 mg. Fe

approaches the theoretical value of 22·4 ml. much more closely after reduction with Ti than before. In Table 4 are given some characteristic figures. These are consistent with the hypothesis that some of the iron in horse blood is present in the ferric state, and that it does not combine with oxygen until it has been reduced to the ferrous condition. Similar results have been obtained with human blood, although they are hardly conclusive, since the initial differences between ferrohaemoglobin and total haemoglobin are so much smaller.

Table 4. *Comparison of oxygen capacity of horse blood with its iron content*

Horse	ml. O_2 bound by 55·84 mg. Fe: Before reduction	After reduction
2	16·5	22·1
3	21·2	21·9
4	17·6	21·4
14	21·3	22·6
15	19·4	22·2
16	19·5	21·9

It has, moreover, been shown by Campbell & Morgan (1939) and by Cox & Wendel (1942) that ferrihaemoglobin formed in blood experimentally or during sulphonamide therapy may disappear spontaneously when the blood is shed. During the present work several opportunities have occurred for checking the oxygen capacity of horse blood for hours and even days after withdrawal. The value has been found to increase until it approaches closely that obtained from the fresh blood after reduction with 'titanous citrate'. For example, horse blood no. 15 showed originally an oxygen capacity of 15·09 vol. % (mean of three estimations made between 30 and 75 min. after withdrawal), while, after 30 hr. at 2°, the value had risen to 17·24 vol. %. The total haemoglobin corresponded initially to an oxygen capacity of 17·28 vol. %, and remained virtually unchanged. Although this experiment was done at the temperature of the refrigerator, it has been found that increasing the temperature up to 15, 25 or 37° has no apparent effect on the increase in oxygen capacity. Neither has the addition of glucose to the blood any influence, a finding which seems related to that of Cox & Wendel (1942) in their study of experimental ferrihaemoglobinaemia. Similar but less striking results have again been obtained with human blood.

Hence, in spite of the absence of direct spectroscopic evidence, it is difficult to escape the conclusion that most samples of horse blood, and a fair proportion of human blood samples, behave as if they contain a certain amount of ferrihaemoglobin. Hardly more than traces have been found in human blood, but horse blood sometimes contains amounts of the order of 20 % of the total haemoglobin present. In all cases, however, the ferrihaemoglobin disappears when the blood is kept for some hours or a day or two.

SUMMARY

1. A modification is described of the Conant-Scott-Douglass technique for the determination of total haemoglobin (ferrohaemoglobin plus ferrihaemoglobin, but excluding carbon monoxide haemoglobin).

2. In 21 out of 38 normal human bloods the total haemoglobin has been found to exceed the ferrohaemoglobin by 1·5 % or more.

3. In 14 out of 17 normal horse bloods the total haemoglobin has been found to exceed the ferrohaemoglobin by more than 1·5 %. In most cases the differences are far greater than those found in human blood.

4. Circumstantial evidence is presented which tends to confirm the hypothesis that the differences are due to the presence of ferrihaemoglobin.

The author is indebted to Prof. Henry Dryerre for encouragement and advice, and to Mr J. W. Burgess, M.R.C.V.S., for assistance in obtaining samples of horse blood. A part of this work was reported in an oral communication to the Physiological Society at Edinburgh in July 1943.

REFERENCES

Ammundsen, E. (1941). *J. biol. Chem.* **138**, 563.
Campbell, D. & Morgan, T. N. (1939). *Lancet*, **2**, 123.
Conant, J. B. & Fieser, L. F. (1924–5). *J. biol. Chem.* **62**, 623.
—— Scott, N. D. & Douglass, W. F. (1928). *J. biol. Chem.* **76**, 223.
Conn, J. B., Kistiakowsky, G. B. & Roberts, R. M. (1940). *J. Amer. chem. Soc.* **62**, 1895.
Coryell, C. D., Stitt, F. & Pauling, L. (1937). *J. Amer. chem. Soc.* **59**, 633.
Cox, W. W. & Wendel, W. B. (1942). *J. biol. Chem.* **143**, 331.
Havemann, R., Jung, F. & Issekutz, B. v. (1939). *Biochem. Z.* **301**, 116.
Lemberg, R., Legge, J. W. & Lockwood, W. H. (1941). *Biochem. J.* **35**, 328.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, vol. **2**, *Methods*. London: Baillière, Tindall and Cox.
Ramsay, W. N. M. (1944). *Biochem. J.* **38**, 467.
Sendroy, J., Jr., Dillon, R. T. & Van Slyke, D. D. (1934). *J. biol. Chem.* **105**, 897.
Van Slyke, D. D. & Neill, J. M. (1924). *J. biol. Chem.* **61**, 523.
Warburg, O., Kubowitz, F. & Christian, W. (1930*a*). *Biochem. Z.* **221**, 494.
—— —— —— (1930*b*). *Biochem. Z.* **227**, 245.
Wendel, W. B. (1938). *J. lab. clin. Med.* **24**, 96.

The Chemistry of Growth and the Food Value of the Common Eel (*Anguilla anguilla* (L.))

By R. A. McCANCE, *Department of Medicine, Cambridge*

(*Received* 24 *August* 1944)

As an article of diet the eel must have a literature dating back for centuries, and although its scientific history cannot claim to go back quite so far, it is nevertheless full of interest. Its life cycle has been shown to include two of the most remarkable migrations known to man. Many excellent investigations, beginning with the famous ones of Paul Bert, have been devoted to the way in which it regulates its osmotic pressure during its passage from inland waters to the sea. A few scattered but, on the whole, incomplete data are available on its composition at maturity and during growth. No study of the kind now to be described appears, however, to have been made.

METHODS

The eels were obtained through the Freshwater Biological Association of the British Empire and were all caught in the north of England. The elvers, which were obtained in 1943 and 1944, had been kept in fresh water for a few weeks before they were sent to Cambridge. The 'snigs', i.e. the small yellow eels weighing about 10 g. apiece, were taken in the years 1943 and 1944 in the river Wyre, Lancs. The yellow eels were caught in 1943 on lines in the Windermere catchment area, and the silver eels in the same year on migration from this area to the sea. The eels were sent to Cambridge packed in grass or weed and the majority were alive on arrival. Yellow eels and the snig eels were obtained in the early summer and again in the autumn, but the elvers and silver eels only when they were migrating. On arrival at Cambridge the eels were measured and weighed. Elvers and one batch of snigs were analyzed whole, but the second and third batches of snigs, and all the larger eels were skinned and dissected with stainless steel knives and scissors. The soft parts were then cut up into small enough pieces to allow duplicate samples, generally of 50 g., to be weighed out for mineral analysis. The remainder was usually minced to facilitate the sampling for nitrogen and fat. At first only muscular tissue was analyzed, although the skins, and the head and guts, were weighed: later the skins, heads and organs were also analyzed so that the composition of the whole fish might be computed. It was, however, impossible to sample the heads and bony parts without mincing them, and hence without running a very serious risk of contaminating them with iron. In all, 51 snigs, 30 yellow eels of assorted sizes and 30 silver eels have been analyzed. The silver eels varied very much in size but were all females. Batches of 4–14 animals have been handled together according to their size and to the numbers received, and their tissues pooled for analysis.

The chemical methods have in the main been those described by McCance & Shipp (1933) or by McCance, Widdowson & Shackleton (1936). Sodium was precipitated as described by these authors and the precipitate titrated with 0·02 N-NaOH (Weinbach, 1935). Water was determined by drying to constant weight at 95–100°. Protein has been taken as N × 6·25. The vitamins were determined by Drs T. Moore, E. Kodicek and Y. L. Wang at the Dunn Nutritional Laboratory, Cambridge. Vitamin A was assayed by the antimony trichloride method, aneurin by the procedure of Harris & Wang (1941, 1942), nicotinic acid by the *p*-aminoacetophenone method (Kodicek, unpublished), and riboflavin by a fluorimetric method (Kodicek & Wang, unpublished).

RESULTS

The chemical composition of whole eels

Table 1 gives the percentage composition of animals at four stages of their development. The elvers were of the order of 0·25 g. in weight, and the amounts of water, N and fat found in them were of the same magnitude as those given by previous analysts. Nitrogen, for example, has been reported to vary from 1·88 to 2·27% and fat from 2·3 to 4·17% (Vaillant, 1903; Reuss & Weinland, 1913; Boucher-Firly, 1935). The snigs averaged 9·4 g. The yellow eels analyzed for Table 1 averaged 197 g., but there is reason to believe (see later and Table 4) that eels of about 100 g. in weight would have had very much the same composition. The figures given for the silver eels are the means of one batch which averaged 197 g. and another which worked out at 1400 g. Size made little difference to their percentage composition. It is at once evident that development involved fundamental changes. There was a progressive and continuous fall in the percentage of water. Between the elver stage and the snig stage this was associated with a considerable increase in protein and a trifling one in fat. Reuss & Weinland (1913) detected these changes in elvers which had been kept and fed in captivity for only 50 days. Such alterations of chemical structure are characteristic of the early growth of many animals and are very well shown by mammals up to and for a variable time after birth (Reuss & Weinland, 1913; Shohl, 1939). As the eels grew and developed further, fat accumulated in them, and this fat displaced both water and protein. Hence the percentage of water continued to fall but with it now the percentage of protein. At maturity, i.e. in the silver eels, these changes were seen in their most exaggerated form for the animals were then loaded

with fat, the protein had fallen to 15·6 % and the water to 59·7 %. Nevertheless, expressed on a fat-free basis there had been little change since the snig stage in the amount of protein in their bodies, or in the water protein ratios, and this is typical of the later stages of mammalian growth (Moulton, 1923; Shohl, 1939). The eel is a fat fish but by no means a very fat animal. The full-term baby contains about 12 % of fat, most of which has been accumulated in the last 8 weeks of foetal life; a normal man is reckoned to be about 18 % fat, a woman would be more, say 25 %—figures quite comparable with the eel. Pigs fatten up easily from about 1·2 % at birth (personal observations) to 11 % of fat at 75 days old and 42 % at 264 days (Ellis & Hankins, 1925).

Table 1. *The composition of eels at various stages of their growth and development*

Eel type	Water (g./100 g.)	Protein (g./100 g.)	Fat (g./100 g.)	Na (mg./100 g.)	K (mg./100 g.)	Ca (mg./100 g.)	Mg (mg./100 g.)	Fe (mg./100 g.)	P (mg./100 g.)	Cl (mg./100 g.)	N (g./100 g.)
Elvers	81·8	12·6	2·19	67	230	515	31·0	4·00	440	55·4	2·02
Snigs	76·5	19·0	2·50	110	302	610	28·0	1·80	475	52·0	3·04
Yellow eels	70·8	17·2	10·60	129	247	411	23·1	1·33	419	92·6	2·75
Silver eels	59·7	15·6	23·20	92	207	323	18·0	1·83	313	79·0	2·51

For osmotic reasons the concentrations of Na and K must be considered together with those of water, for changes in the one without the other would involve violations of the animals' osmotic pressures. It will be seen that there was a rise in the concentrations of Na and K in passing from the elver to the snig stage. This was associated, as already mentioned, with a fall in the percentage of water, and must have meant a rise in osmotic pressure, and it is in keeping with the facts (*a*) that the elvers were analyzed after they had been in fresh water for a little time, (*b*) that in elvers the power to regulate osmotic pressure is not yet fully developed (Boucher-Firly, 1935). The fall in water as the eels grew from the snig stage and matured was accompanied by a fall in the sum of the milli-equivalents of Na + K in the animals. If twice the sum of the concentrations (in m.equiv./l. of body water) of Na and K be taken as a measure of osmotic activity, the data for osmotic activity given in Table 2 can be calculated from the figures shown in Table 1. It will be seen that the osmotic pressure in the elvers was well below that in the older eels, and it is interesting to note that the figures found for the latter agreed quite well with the one for human serum. This, of course, was to be expected from what is known of the osmotic pressures of mature eels and other vertebrates.

Table 2. *Osmolar concentrations in eels*

Eel types	mM. of osmotically active material/l. of tissue water
Elvers	215
Snigs	328
Yellow eels	337
Silver eels	307
Human serum (on the same basis)	318

The figures for the alkaline earth metals deserve some consideration. After the eel has been in fresh water for about 3 years, calcium begins to be deposited not only in the bones (as in mammals) but also in the scales which are buried in the skin. Hence, whereas 99 % of the body calcium of man is in the bones (Shohl, 1939), and this is probably true also of the elvers and young snigs, only 86 % of the calcium in the bodies of yellow eels was found in the bones and 75 % of that in the silver eels. Nevertheless, the amount in the skin is a relatively small and constant fraction of the total calcium of the body (see later, and Table 4), so that gross accretions in body calcium may be considered, as in the mammal, to represent bony growth. The rise, therefore, in the percentage of calcium in the body between the elver and the snig stage may certainly be assumed to be associated with the formation of the bones and the deposition of calcium therein at a greater rate than the general increase in body weight. A changing relationship of this type was detected by Reuss & Weinland (1913) in elvers, and characterizes the early growth of all vertebrates. As the snigs developed into larger yellow eels and finally matured to silver eels the percentage of calcium fell. To a limited extent this was due to the deposition of fat, but it is at these later stages that the rate at which calcium is deposited in the eel (and probably in all fish without a high degree of scale development) diverges from the rate at which it is deposited in a land animal. To show this Table 3 has been compiled from Shohl (1939) and from Duckworth & Warnock (1942). It shows, in g. or mg./100 g., the fat, calcium and magnesium in a human being at various ages. The diverging trends for calcium in man and eel are due to the different degrees of bony development required by aquatic and land animals. It would be interesting to have similar data for aquatic mammals.

Comparative bony development also explains why the eel has a much smaller percentage of 'total ash' in its body than a land animal. Thus the pig and man have about 3 % of ash in their fat-free bodies

Table 3. *The concentration of fat, calcium and magnesium in human bodies*

Age	Fat (%)	Ca (mg./100 g. of whole body)	Mg (mg./100 g. of whole body)
Foetus: 4 months	0·5	340	18·0
6 months	2·5	605	20·5
Premature baby, 7 months	3·0	726	21·4
Full-term baby	12·0	810	23·7
Child, 10 years	15·0	2000	—
Adult	18·0	2300	29·5

(This table is based on the data of Shohl (1939) and Duckworth & Warnock (1942).)

at birth, and this percentage rises with development (Moulton, 1923; Ellis & Hankins, 1925). If the percentage of total ash (and therefore of calcium) in a land animal appears to fall in the later stages of growth, as it has been shown to do in the pig (Ellis & Hankins, 1925), it can be accounted for entirely by deposition of fat. By calculating the elements in Table 1 as oxides or chlorides, the snigs can be shown to have had only 2·6 % of ash in their bodies, and this percentage fell with growth so that the silver eels contained only 2·1 % of ash on a fat-free basis.

In the eel (Table 1) the percentage of Mg in the body was found to fall from the elver stage to Ca and Mg to vary together. In man they do so, and in the eel they do so too after the snig stage. The large amounts of Mg in the elvers, however, can hardly be attributed to their bony development, for they contained less Ca than the snigs, but it may be associated with the fact that these animals have only recently left sea water, which is rich in Mg salts.

The amount of P in the body depends upon the degree of bony development and the figures, therefore, follow those for calcium, but the changes are smaller because in the eel there is relatively more P outside the bones and skin, and, therefore, the bony changes are to some extent 'buffered' by the stable composition of the tissues.

The figures for Na, K, Ca and Mg can be regarded in another way. These four cations together constitute so nearly the whole of the total base of the body that they may be assumed to do so. If their sum be taken as 100, the results shown graphically in Fig. 1 are obtained, and the corresponding data for a human being taken from Shohl (1939) and Duckworth & Warnock (1942) are given beside them. In the earlier stages of development both young animals would have shown a progressive increment in the percentage of calcium. The divergence later is well shown in the diagram.

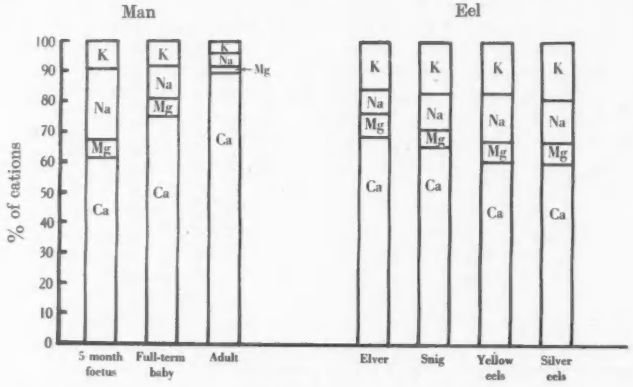


Fig. 1. A comparison of the percentage 'make-up' of the total base in the human body and in the eel at various stages of development.

maturity: in man (Table 3) it will be seen to rise throughout the period of growth. According to Duckworth & Warnock (1942), 80 % of the Mg in a man is in the bones, and the corresponding percentage in the eel, though smaller, must be considerable. Consequently, in both animals, one would expect the extent to which the skeleton was developed and mineralized to determine the percentage of Mg in the body, and the percentages of

The striking thing about the figures for iron are the large amounts of this element found in the elver. If this accumulation of iron could be shown to have any biological significance it would be very interesting. Mammals are born with an accumulation of iron in their bodies, and this has been regarded rather teleologically as a provision of nature for the low iron intakes of the suckling period. It may be, however, that storage of iron is

characteristic of some phase in the development of all vertebrates.

The composition of tissues and organs

Table 4 shows the composition of the muscular tissues of eels at various stages of development, of the livers and skins taken from yellow and from silver eels, and of the heads, guts, livers and bones taken together.

Table 4. *The percentage composition of eel organs*

Eel type and average weight	Water (g.)	Protein (g.)	Fat (g.)	Na (mg.)	K (mg.)	Ca (mg.)	Mg (mg.)	Fe (mg.)	P (mg.)	Cl (mg.)
Muscle:										
Snigs 9·4 g.	78·0	18·0	3·1	76	290	26·0	18·7	0·50	210	74
Yellow eels 103·0 g.	71·0	17·0	11·2	62	281	21·5	20·4	0·90	226	50
Yellow eels 193·0 g.	71·5	16·2	11·4	115	251	15·5	17·5	0·53	220	63
Silver eels 197·0 g.	58·3	14·0	26·7	77	190	16·3	14·3	1·17	190	55
Silver eels 333·0 g.	53·8	15·3	29·8	85	236	11·6	14·4	0·76	192	111
Silver eels 678·0 g.	59·1	14·6	26·4	97	191	13·3	14·9	0·54	190	62
Silver eels 1400·0 g.	57·0	13·5	28·3	50	243	9·3	13·9	0·53	197	49
Liver:										
Yellow eels 188·0 g.	78·7	14·3	4·7	165	221	6·5	13·5	25·00	277	—
Silver eels 333·0 g.	77·9	13·2	6·5	203	196	8·9	11·4	105·00	244	193
Skin:										
Yellow eels 188·0 g.	—	22·8	5·2	105	126	468·0	17·2	0·86	350	—
Yellow eels 197·0 g.	68·0	25·2	5·2	147	183	585·0	19·1	2·67*	445	184
Silver eels 197·0 g.	66·5	25·8	6·2	144	148	670·0	19·4	5·25*	405	156
Silver eels 333·0 g.	—	27·5	5·2	194	121	730·0	16·2	1·05	439	—
Heads, guts, livers, bones together:										
Yellow eels 197·0 g.	69·7	17·1	9·3	168	224	1240·0	35·4	3·1*	805	129
Silver eels 197·0 g.	59·8	16·0	16·9	135	204	1230·0	35·4	3·4*	715	93
Silver eels 1400·0 g.	60·1	15·7	19·1	150	174	1060·0	26·5	6·7*	515	123

* Minced.

Muscular tissue constitutes about two-thirds of the total weight, and forms the main fat depot of the animal. It will be seen from the table that yellow eels of rather varying sizes tended to have about 11 % of fat in their muscles, whereas the silver eels had 26–30 %. Each figure given in Table 4 is the average of at least four animals, and there is no doubt that the individuals varied very much on either side of these means. Boucher-Firly (1935) found that the muscles of small eels weighing 20–25 g. contained 4·5–21·4 % of fat and averaged 13·5 %. These figures seem very high for eels of that size, but the larger animals must accumulate fat in their muscles preparatory to their metamorphosis and migration, and so very fat ones must occasionally be caught, but the number of yellow eels with more than 15 % of fat in their muscles is probably only a very small proportion of the total eel population of our rivers and lakes. Be that as it may, these results do not support Lovern's (1938) contention that the amount of fat in an eel can be foretold from its length. Lovern did not state whether his eels were all immature yellow animals, but from the nature of his experiments it may be assumed that they were. Boucher-Firly's (1935) results are also in conflict with those of Lovern (1938), and it is worth noting that by 1940 Lovern was himself beginning to find exceptions to his 'fat-length' generalization. Muscle tissues, like the whole animals, show a fall in both water and protein with each increment of fat, and also a relatively constant water-protein ratio whatever the level of fat (Moulton, 1923). Mammalian work suggests that some muscles would be consistently fatter than others, and that some might accumulate fat at an earlier age than others, but no information about this is available.

The eel was one of the animals which Katz (1896) included in his classical survey of the minerals to be found in muscular tissue. He analyzed two large eels and his figures for Na, K, P and Cl are near those given here for the largest silver eels. In the present study the sodium in the muscles varied from 48 to 115 and the potassium from 190 to 281 mg./100 g. No doubt the individuals varied much more. The ratio of potassium to nitrogen also exhibited rather wide variations. The chemical evidence, therefore, suggests that the concentrations of minerals in the cellular and extracellular compartments of the muscle mass of the eel may not be so constant as they are in the mammal. Alternately the cellular-extracellular ratio of the muscle mass may vary considerably. Table 4 shows that there was no consistent change in the Na/K ratio as the animals put on fat, so that the fat may be regarded simply as 'diluting' the whole of the watery phase of the muscles.

The figures given for muscular calcium in Table 4 are probably in all cases a little too high, due to the

inclusion of minute bones. They are, however, lower than those of Katz (1896). The figures for Mg bear some relationship to the protein, which is to be expected since the magnesium is almost confined to the cells. The P varies in a similar way. The Fe is on the low side, even for fish muscle.

The livers of the yellow eels averaged 1·5 % of the weights of the whole animals, and those of the silver eels 2 %. They contained rather more water and less protein than the muscles of the corresponding animals and considerably less fat. Boucher-Firly (1935) found a rather higher percentage of fat in the livers of both yellow and silver animals, but again considerably less than in the muscles. The livers resemble those of mammals in containing little calcium and less magnesium than the muscle (McCance & Widdowson, 1940). This difference is probably associated with function and may be common to all vertebrates. The Fe was exceedingly high, particularly in the silver animals, and Fe storage must be one of the functions of the organ as it is in the higher vertebrates. It may be that the accumulation of Fe in the liver is as much a part of the preparation for migration as the storage of fat in the muscle. The livers contained more sodium and chlorine than the muscles.

The skin, excluding that of the head, constituted about 8 % of the weight of the yellow eels, and 10–12 % of that of the silver eels. It contained little fat, whatever the fat reserves of the animals, but a very high percentage of protein, twice as much in fact as the liver in the case of the silver eels. The sodium and chlorine were relatively high and the potassium low. The high figures for calcium and phosphorus are due to the calcified scales embedded in the skin, and these scales certainly contained some of the Mg. The true figure for Fe is probably of the order of 1 mg./100 g.

The heads, guts, livers and bones together constituted 25–28 % of the yellow eels and 20–22 % of the silver eels. These figures include a certain amount of muscular tissue which was left on the vertebrae and small bones. The vertebrae themselves, when carefully cleaned, came to between 4 and 5 % of the animals' weight, and with the bones of the head would come to a little more. The figures for minerals require little comment, except to note the very high values for Ca and P. The bones in all probability contained enough Mg to account for the high figures of 26–35 mg./100 g. in the mixed organs. The figures for Fe may be too high owing to the tissues having been contaminated while they were being minced, but some blood was always lost while the animals were being dissected and gutted, and the two errors would have tended to cancel themselves out.

The sum of the sodium and potassium salts in each litre of tissue water has been determined in each of these organs. In general the data thus obtained were similar to those for the whole animals (see Table 2), but in any one batch the mixture of bones, heads, guts and livers has always given a figure higher than that of the muscles or skin. The explanation for this may be that fish bones, as mammalian bones, contain a certain amount of sodium adsorbed on to the crystals of hydroxy-apatite (Hodge, Koss, Ginn, Falkenheim, Gavett, Fowler, Thomas, Bonner & Dessauer, 1943). Such sodium would be osmotically inactive.

Growth increments

Table 5 shows the length, weight, approximate age and the total amounts of protein, fat and minerals in eels at various stages in their development. This gives at a glance their rate of chemical growth. The total caloric value of these animals may be calculated from their organic composition, and if their metabolic rates could have been determined at representative times of year one could have calculated at once the calorific value of the food they had eaten at each stage of their lives. In a similar way, if the amount and composition of the

Table 5. *The weights of protein, fat and mineral elements in eels at various stages of their growth and development*

Eel type	Length (cm.)	Weight (g.)	Approx. age* (years)	Water (g./eel)	Protein (g./eel)	Fat (g./eel)	Na (mg./eel)	K (mg./eel)	Ca (mg./eel)	Mg (mg./eel)	Fe (mg./eel)	P (mg./eel)	Cl (mg./eel)
Elvers	7·3	0·25	2– 3	0·205	0·032	0·006	0·17	0·58	1·29	0·08	0·01	0·11	0·14
Snigs	22·6	9·4	6– 7	7·100	1·890	0·290	10·30	27·2	55·2	2·90	0·153	44·60	6·95
Yellow eels:													
(1)	38·0	103	9–10	73·6	17·100	10·300	136	231	372	27·50	1·22	371	84·4
(2)	49·5	197	12–13	140	33·900	20·900	254	486	810	45·60	2·62	825	183
Silver eels:													
(1)	49·3	197	10–12	118	31·100	43·500	201	373	720	38·40	4·23	617	149
(2)	57·0	333	13–14	195	52·400	84·000	356	716	1030	59·30	5·42	1090	398
(3)	70·2	678	16–18	409	107·000	152·000	775	1260	2100	124·00	12·30	2250	587
(4)	86·6	1400	18–21	810	216·000	340·000	1160	3010	3920	232·00	36·30	4100	1140

* From time of hatching.

food eaten were known, one could calculate the percentage of the minerals in it which had been retained. The figures require no particular comment but there is one point of interest which must be discussed. It will be noted that the larger yellow eels and the smallest silver eels averaged exactly the same weight. This hypothetical yellow eel contained a little more protein, but much less fat than the silver eel, more water, Na and K, however, and also more Ca, Mg and P. This comparison demonstrates the complexities of the change from yellow to silver. Unless the process is accompanied by a loss of Ca and P, the figures for these elements suggest that a yellow eel weighing, say 200 g., cannot turn into a silver eel weighing less than 250 g.

As all sea fish, they would no doubt be rich in iodine. Eels are usually eaten stewed, and this process leads to a loss of nutrients (McCance & Shipp, 1933) unless the gravy is eaten. When 300 g. of silver eels in portions weighing about 50 g. were stewed in 150 ml. of water for 20 min., the losses (%) were as follows: weight 25, protein 7·8, fat 12·4, Na 28·0, K 30·1, Ca 14·5, Mg 22·6, Fe trace, P 22·0, Cl 31·0. The amounts of these losses and the ratios of one to another are quite characteristic of a flesh food (McCance & Shipp, 1933). The composition of stewed eels may be computed from the above data by those who desire the information.

Table 6 gives the amounts of vitamin A, aneurin, riboflavin and nicotinic acid found in these eels.

Table 6. *Vitamins in uncooked eels*

The figures refer to muscle tissue only except in the case of elvers, which were analyzed whole.

		Yellow eels. Average weights of groups analyzed				Silver eels. Average weights of groups analyzed			
		June catches		Autumn catches					
	Elvers	103 g.	256 g.	188 g.	197 g.	197 g.	333 g.	678 g.	1400 g.
Vitamin A, i.u./100 g.	0	1500·00	300·00	2400·00	1500·00	900·00	3000·00	1800·00	1500·0
Aneurin, μg./g.	1·60	2·10	2·00	1·80	1·80	1·80	5·40	2·60	2·6
Riboflavin, μg./g.	2·93	3·82	5·16	2·60	2·50	3·00	4·20	3·20	2·9
Nicotinic acid, μg./g.	9·00	7·65	6·55	14·10	24·20	7·80	15·50	24·80	7·8

The food value of the eel

Elvers are considered a great delicacy in France and Spain. They have not got such a reputation over the whole of England, but they are caught in large numbers in the Severn and regarded as first-class fare all over Gloucestershire. Eels of the snig size, i.e. 20–25 cm. in length, are not eaten much if at all in this country, but, according to Vaillant (1903), they are much esteemed in France under the name of 'anguilles poulettes'. Of the larger animals, only the muscular tissues are eaten as a rule, although no doubt the livers would be very nutritious. The figures given by McCance & Widdowson (1940) refer to a mixture of stewed yellow and silver eels. The composition of the muscles of uncooked yellow and silver eels may be taken from Table 4, and the amounts of edible material and waste are given in the text. It will be appreciated that any food containing 27% of fat will have a high calorific value, and 100 g. of the flesh of a silver eel would provide the body with 300 cal., a figure which is probably higher than that provided by any other fish, and which compares favourably with the value even of the concentrated foods. The proteins must be of high biological value, but the mature animals cannot be regarded as supplying much Ca or Fe. The elvers are clearly very rich in Ca and would be as good a source of Fe as beef. Muscular tissue only was analyzed, except in the case of the elvers, which were treated whole. It will be noted that the figures for aneurin and riboflavin were relatively constant. Those for nicotinic acid were less so and those for vitamin A were very variable, and the results bear no obvious relationship to the season of the year or to the amount of fat in the animals. The muscles are, however, a rich source of A, and very much richer than the herring (Bacharach, Cruickshank, Henry, Kon, Lovern, Moore & Morton, 1942).

SUMMARY

1. Data have been presented: (*a*) For the composition of elvers after some weeks in fresh water, and of yellow and silver eels of various sizes. (*b*) For the weight and composition of eel organs. (*c*) For the food value of eels and for their losses on cooking.

2. On a percentage basis, elvers contain less protein, fat, Na and K salts, and more water than older eels, and they must have a lower osmotic pressure. They are rich in iron and magnesium.

3. The next few years are characterized by an increase in the percentage of protein, Na and K and Ca salts. The percentages of water, iron and magnesium fall.

4. In the later years of growth and in the preparation for migration, fat is gradually deposited.

particularly in the muscles, with a corresponding fall in the percentage of protein, water, Na and K salts. The fall in Ca is greater than can be accounted for in this way.

5. The liver of a silver eel may contain over 100 mg. of iron/100 g. wet weight. There may be over 25 % of protein in the skin.

6. The above, and other chemical changes of growth have been correlated with events taking place in the body and with similar and dissimilar changes in the chemistry and growth of the higher vertebrates.

7. 100 g. of the flesh of a silver eel would provide 15 g. of 'first class' protein, and 300 cal. From the point of view of food, elvers must be regarded as a good source of Ca, P and Fe.

The author is indebted to Miss Frost and to other members of the Freshwater Biological Association for supplying the animals, and for their advice about them; to the staff of the Nutrition Laboratory, Cambridge, for the vitamin assays, and to Miss D. N Graves and to Dr E. M. Widdowson for their help with the chemical determinations. The work was the outcome of a request from Dr E. B. Worthington for more information about the food value of the eel.

REFERENCES

Bacharach, A. L., Cruickshank, E. M., Henry, K. M., Kon, S. K., Lovern, J. A., Moore, T. & Morton, R. A. (1942). *Brit. med. J.* **2**, 691.

Boucher-Firly, S. (1935). *Ann. Inst. océanogr. Monaco*, N.S. **15**, 217.

Duckworth, J. & Warnock, G. M. (1942). *Nutr. Abstr. Rev.* **12**, 167.

Ellis, N. R. & Hankins, O. G. (1925). *J. biol. Chem.* **66**, 101.

Harris, L. J. & Wang, Y. L. (1941). *Biochem. J.* **35**, 1050.

—— —— (1942). *Chem. Ind.* **61**, 27.

Hodge, H. C., Koss, W. F., Ginn, J. T., Falkenheim, M., Gavett, E., Fowler, R. C., Thomas, I., Bonner, J. F. & Dessauer, G. (1943). *J. biol. Chem.* **148**, 321.

Katz, J. (1896). *Pflüg. Arch. ges. Physiol.* **63**, 1.

Lovern, J. A. (1938). *Biochem. J.* **32**, 1214.

—— (1940). *Biochem. J.* **34**, 704.

McCance, R. A. & Shipp, H. L. (1933). *Spec. Rep. Ser. Med. Res. Coun., Lond.*, no. 187.

—— & Widdowson, E. M. (1940). *Spec. Rep. Ser. Med. Res. Coun., Lond.*, no. 235.

—— —— & Shackleton, L. R. B. (1936). *Spec. Rep. Ser. Med. Res. Coun., Lond.*, no. 213.

Moulton, C. R. (1923). *J. biol. Chem.* **57**, 79.

Reuss, H. & Weinland, E. (1913). *Z. Biol.* **59**, N.F. **41**, 283.

Shohl, A. T. (1939). *Mineral Metabolism.* New York: Reinhold Publishing Co.

Vaillant, L. (1903). *C.R. Soc. Biol., Paris*, **55**, 749.

Weinbach, A. P. (1935). *J. biol. Chem.* **110**, 95.

Fat Formation in *Torulopsis lipofera*

By A. KLEINZELLER, *Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, Cambridge*

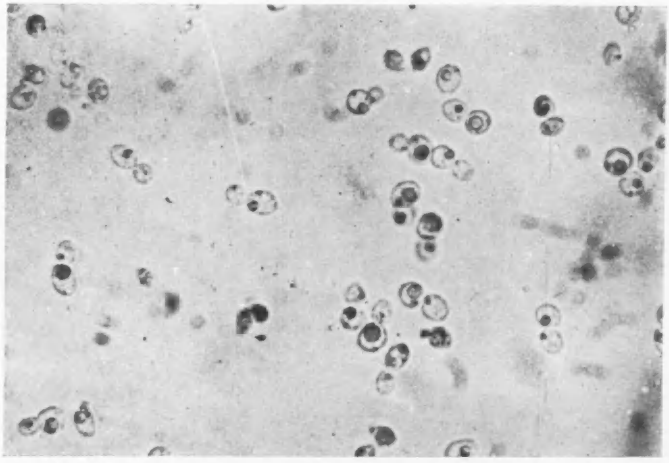
(*Received* 28 *August* 1944)

Several micro-organisms are known to be capable of storing considerable quantities of fat in their cells (Fink, Haehn & Hoerburger, 1937; Prescott & Dunn, 1940). The formation of fat in these organisms is either closely connected with growth, or very slow. These organisms therefore are not suitable for an investigation of the mechanism of fat synthesis. In the present paper the metabolism of *Torulopsis lipofera* (den Dooren de Jongh, 1926) was investigated. This organism lends itself particularly well for a study of fat synthesis because of its easy growth and rapid conversion of carbohydrate into fat.

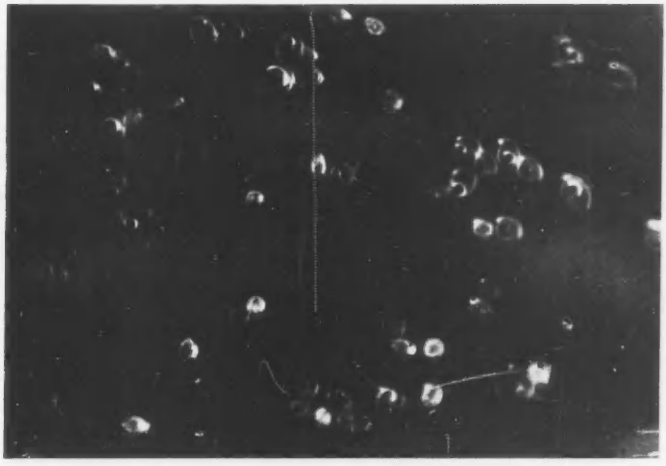
MORPHOLOGY AND GROWTH OF *TORULOPSIS LIPOFERA*

T. lipofera was first described by den Dooren de Jongh (1926) as an asporogenous soil yeast, capable of storing large droplets of fat in its cells. Lodder (1934) investigated the morphological properties and biochemical behaviour of this yeast somewhat more in detail. The strain of *T. lipofera* employed in the present study was obtained from the National Collection of Type Cultures. The yeast grows well on flour-wort-agar, or on simple inorganic medium (Stephenson, 1939) with 0·05 % marmite and carbohydrate, or on molasses containing NH_4, K and Mg salts. Morphologically the cultures consist principally of oval cells containing several droplets of fat and some round cells with a very large fat droplet filling most of the cell volume (see Pl. 4). These two types of cells do not appear to represent different strains, because on single-cell isolation of the oval type of cells round cells also were found in subsequent subcultures.

Growth of T. lipofera. The growth conditions were studied by measuring turbidimetrically the growth rate of the yeast cultivated at room temperature



A



B

Microphotographs of *T. lipofera*. A, magnification 500 ×; B, dark-ground illumination, magnification 600 ×.

Table 1. *Yield of cells of* T. lipofera

Growth medium	Carbohydrate used; calc. as glucose (g.)	Yeast formed (g.)	Fat (fatty acids and unsaponifiable) (% dry wt.)	Ash (%)	Yeast as % of sugar used
Inorganic medium + marmite (0·05%) + glucose (2%)	9·41	3·92	37·2	—	42·0
Molasses + salts	51·4	24·5	43·0	4·9	47·7

in the inorganic medium (Stephenson, 1939) plus 0·05% marmite and 1–2% carbohydrate.

Approximately 300 ml. of the sterile medium were placed into each of a number of sterile glass tubes 75 cm. long and 3·5 cm. wide, provided with an outlet for withdrawing samples. Thorough aeration was achieved by passing sterile air through porous aerating blocks at a rate of 300 ml./min.; this produces bubbles of about 10μ diameter. The medium was then inoculated and the growth rate at room temperature (16–20°) was measured every 12 hr. for a period of 72–96 hr. After a lag period of about 6 hr. the growth was logarithmic.

Using this method the following different conditions had no effect on the growth rate: variation of pH between 4·0–6·0; addition of tryptic digest of casein (0·05–0·1% N) or asparagine 0·01%; variation of the glucose concentration between 0·5 and 2% and of the NH_4 between 0·03 and 0·12M; replacement of the glucose by an equivalent amount of sucrose or molasses. The generation time of the yeast in these experiments was 11–16 hr.

The optimal temperature for the growth of *T. lipofera* is 23–25°. At 30° the yeast grew for 1–2 days and then died off (Fig. 1). Under best conditions,

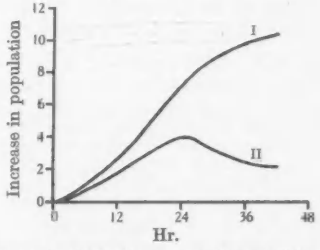


Fig. 1. Growth of *T. lipofera* at 25 and 30°. Medium: molasses containing 2% carbohydrate and NH_4, K, and Mg salts. I, 25°; II, 30°.

i.e. with good aeration and strict control of temperature, pH, N and carbohydrate (molasses) content, the generation time was 5½–5½ hr.; this growth is relatively slow when compared with that of some other yeasts, e.g. *T. utilis* (Thaysen & Morris, 1943). The dry weight of yeast obtained was 42–48% of the carbohydrate used (Table 1); a remarkably high content of fatty material (unsaponifiable and fatty acids) is obtained.

Nitrogen and vitamin content of T. lipofera. Two samples of freeze-dried yeast were examined for N and vitamin content. Sample I was grown on glucose and contained 28·1% fatty material; Sample II was grown on molasses and contained 18·6% fatty material. Estimations of the vitamins were kindly carried out by Dr Y. L. Wang (aneurin, riboflavin, and nicotinic acid) and by Dr T. Moore (carotene). As shown in Table 2, the content of

Table 2. *Nitrogen and vitamin content of* T. lipofera

	Grown on: Glucose I	Molasses II
Fat (%)	28·1	18·6
Total N (%)	5·43	5·85
Aneurin (μg./g.)	5·8	8·3
Riboflavin (μg./g.)	39	41
Nicotinic acid (μg./g.)	305	230
Carotene (μg./100 g.)	—	10

aneurin, riboflavin and nicotinic acid in *T. lipofera* is of the same order as in other yeasts; the content of carotene is low.

T. lipofera used in the experiments described below was grown at room temperature for 2–3 days in liquid inorganic medium (Stephenson, 1939) plus 0·05% marmite and glucose (1–2%) in the glass tubes described above. The yeast was then spun off, washed twice with 0·1M-NaCl and finally suspended in a small volume of saline. The yeast suspension was kept in an ice-chest and used within 2 days.

EXPERIMENTAL METHODS

1. *The manometric experiments* were carried out in Warburg manometers with conical flasks. A suspension containing 20–25 mg. yeast (dry wt.) in 0·1M-phosphate buffer of varying pH was measured into the main compartment of the flask. The substrates were placed in the side-arm. The volume of the cup contents was brought to 3·0 ml. with 0·1M-phosphate buffer. In experiments where the oxygen uptake was measured, 0·2 ml. 2N-NaOH was placed in the centre well.

The R.Q. was measured by means of the method of Warburg & Yabusoe (1924). As it was subsequently shown that CO_2 inhibits the utilization of glucose and fat formation (see Table 9), the measurements of the R.Q. are of only limited value. In anaerobic experiments nitrogen free from oxygen was used to fill the gas-space and yellow phosphorus was placed in the centre well to absorb traces of oxygen. After thermal equilibrium was reached the oxygen uptake and/or CO_2 output were measured over a period of 2–3 hr.

2. *Glucose* was estimated by the cerimetric method (Miller & Van Slyke, 1936) with Xylene Cyanol FF as indicator. A sample of the suspension was placed in a

25 ml. volumetric flask, 1 ml. of 2N-HCl added to stop any metabolic reactions, and the volume made up to the mark with water. After the yeast had settled a sample of the clear supernatant solution was used directly for the glucose estimation, without deproteinization. Duplicates of glucose estimations agreed within 1%.

3. *Phosphate* was estimated according to Fiske & Subbarow (1925).

Estimation of fat

The method was based on those of Kumagawa & Suto (1908), Lemeland (1923) and Belin (1926). The yeast is saponified with 2·5N-KOH in 98% ethanol, and the soap solution acidified and extracted with ether. The ether extract is dried *in vacuo*, the fatty material redissolved in light petroleum, filtered and estimated either (*a*) by weighing (macro-estimation) or (*b*) by determination of the C content manometrically according to Van Slyke & Folch (1940) (micro-estimation).

(*a*) *Macromethod.* The conditions for the saponification of the yeast were first worked out on a macro scale. Freeze-dried yeast (1 g.) was saponified with 20 ml. ethanolic KOH in a 50 ml. flask provided with an air condenser; the flask was heated on a steam-bath, the ethanolic soap solution diluted with water, the unsaponifiable matter extracted twice with ether, the ether extract washed twice with water, the ether distilled off and the unsaponifiable matter dried and weighed. The soap solution was combined with the washings of the ether extract of the unsaponifiable fraction, acidified with 5N-H_2SO_4, and the fatty acids extracted three or four times with ether. The combined extracts were washed twice with water, the ether distilled off, the dry fatty acids redissolved in light petroleum (b.p. 50–60°), filtered through asbestos, the solvent distilled off and the fatty acids dried *in vacuo* and weighed.

Table 3. *Macro-estimation of fat in* T. lipofera

(2½ hr. saponification.)

	Concentration of KOH (in 80% ethanol)		
	0·8N	2·5N	5N
Unsaponifiable matter (%)	1·43	2·18	1·91
Fatty acids (%)	26·5	25·9	26·2
Mean mol. wt. of fatty acids	—	273·5	273·5
Carbon content of fatty acids (%)	—	75·84	76·19
Iodine value	—	89·6	83·5

As is shown in Table 3 the yeast is completely saponified by ethanolic-KOH (2·5N) in 2½ hr.; the use of ethanolic-KOH (0·8N) gave somewhat low figures for the unsaponifiable fraction. The mean molecular weight of the fatty acids was 268–282, their C content 75·8–76·2%, and their iodine value (Rosenmund & Kuhnhenn, 1923) 55–82 (see also Table 18).

(*b*) *Micromethod.*

Reagents: (1) 2·5N-KOH in 98% ethanol. The ethanol should be distilled over solid NaOH to remove substances forming resins on boiling with KOH. The reagent must be freshly prepared. (2) Ether, dried over Na, and freshly distilled. (3) Light petroleum, b.p. 50–60°, purified as described by Bloor (1929). (4) 50% H_2SO_4 (by vol.). (5) Reagents for macro- or micro-C-estimation (Van Slyke & Folch, 1940).

Apparatus: (1) 15 ml. centrifuge tubes with glass ground jointed air-condenser 30 cm. long. (2) Kutscher-Steudel liquid extractor of approx. 25 ml. capacity with 100 ml. flask. For details see Krebs, Smyth & Evans (1940). (3) Emich's microfilter with porous glass filter and a layer of washed asbestos. The filter must be dry before use. (4) A B19 standard joint cone with side-arm for suction as shown in Fig. 2A. (5) Van Slyke manometric apparatus equipped for C estimations (Van Slyke & Folch, 1940).

All glass apparatus used for fat estimations must be carefully cleaned with hot chromic-sulphuric acid mixture, washed and dried before use.

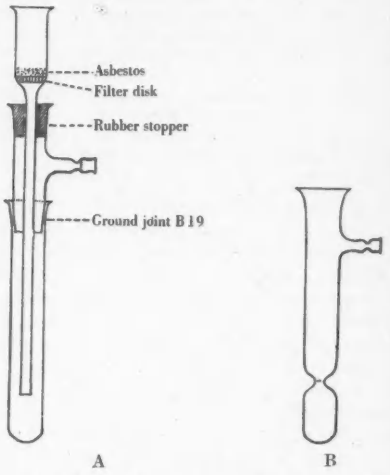


Fig. 2. Filtration of fat. A, filtration into combustion tube; B, calibrated suction tube.

Procedure. The yeast is transferred to the centrifuge tube. In metabolism experiments 10 ml. yeast suspension are run into the tube containing 1 ml. 2N-HCl to stop any metabolic reactions. The suspension is then centrifuged for 5–10 min. at 3000 r.p.m. and the supernatant siphoned off. The yeast is then washed with 4–5 ml. 0·1M-NaCl, centrifuged again and, after removal of the supernatant by suction, ethanolic KOH (4 ml.) and a few grains of clean quartz are placed in the tube, the air condenser fitted, and the yeast saponified by immersing the tube for 2½ hr. in a water-bath at 87–90°. This temperature is sufficient to keep the ethanolic solution boiling gently. After the saponification is completed the condenser is removed, and traces of soap which may have splashed on the joint are washed with 0·5 ml. water into the tube. The ethanol is now carefully evaporated on a water-bath at 85–90°. It was found useful to add a few more grains of quartz before the evaporation in order to prevent undue bumping. The soaps are then quantitatively transferred to the Kutscher-Steudel extractor by pipetting the diluted soap solution and three subsequent washings of the tube and condenser into the inner funnel of the extractor (volume of solution 15–20 ml.). The solution is then acidified with 1 ml. '50% H_2SO_4' and the fatty acids and the unsaponifiable matter are continuously extracted for 1½–2 hr. with a known volume of ether (40–45 ml.). About 12 ml. of ether pass through the

solution per min. The ether in the flask is then distilled off, the residue dried on a vacuum pump at 0·5–1 mm. Hg for 10 min., and 1 ml. light petroleum added. The solution, usually somewhat cloudy, is left standing for 30–60 min. at room temperature in order to allow emulsified resinous material to precipitate (Kumagawa & Suto, 1908). The solution is then filtered through the Emich filter by suction into the combustion tube. Three washings of the flask with 0·5–1 ml. light petroleum ensure quantitative transfer of the fatty material into the combustion tube. The solvent is removed as described by Van Slyke & Folch (1940) and a macro-C-estimation is carried out. The fatty material obtained in this way is generally white or yellowish. It consists of fatty acids and a small amount of unsaponifiable material (see Tables 3 and 18), and can be considered to be free from organic impurities.

If samples of the fatty material solution are to be used for analysis, the light petroleum extract is filtered into calibrated tubes prepared by drawing out suction tubes (conveniently old Thunberg tubes) and calibrating these with Hg after marking them on the narrow part (see Fig. 2B). A sample (1–2 ml. out of 4–6 ml.), after thorough mixing of the contents of the tube, is then taken for micro-C-determination.

As shown in Tables 3 and 18, the unsaponifiable material forms only a small fraction of the fat present (6·8–8·8%). The C content of the fatty acids is 76%. The fatty acid content of the yeast can therefore be obtained with sufficient accuracy by multiplying the C of fat found by the factor 1·32.

A blank analysis on the reagents is carried out, starting from the saponification. In these experiments the blank varied from 0·02 to 0·1 mg. C.

Table 4. *Micro-estimation of fat*

	Recovery of fat	
	(mg.)	(%)
7·65 mg. stearic acid	7·58	99·2
28·68 mg. yeast containing 8·06 mg. fat (macro-estimation)	7·94	98·5

As shown in Table 4, stearic acid is quantitatively recovered after having been treated with the

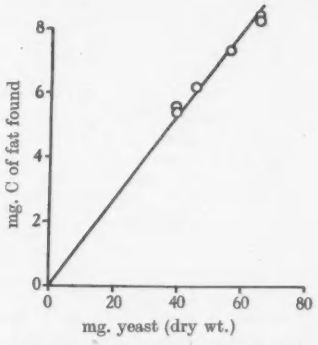


Fig. 3. Micro-estimation of C of fat in *T. lipofera*.

ethanolic KOH, acidified, extracted, filtered, and the C content estimated. The results on yeast agreed with those obtained by the macromethod within 1–3%. Duplicate microdeterminations agreed within 1–2%. The C of fat found increases linearly with increasing amounts of yeast (Fig. 3).

The micromethod described above may be conveniently applied to the determination of fatty material (unsaponifiable and fatty acids) in any tissue after ascertaining the C content of the fatty material, which may vary slightly according to the nature of the fatty acids and the content of unsaponifiable material. If necessary, of course, the unsaponifiable matter and fatty acids could be estimated separately in the usual way.

Procedure for studying fat metabolism

A suspension of yeast in 0·13 M-buffer of the required pH is made up, containing approximately 65 mg. of yeast (dry wt.)/10 ml. Ten ml. of this suspension are then pipetted into 100 ml. conical flasks and 3 ml. additions (substrate and/or water) are added. One ml. of the suspension is—if required—removed immediately for determination of the initial content of substrate. Ten ml. of the contents of one flask are pipetted into a centrifuge tube for the estimation of the initial content of fat. The remaining flasks are then stoppered and shaken in a thermostat at 25° (unless otherwise stated) for 3–9 hr. After removal of a flask from the thermostat, 1 ml. of its contents is taken for analysis of the substrate and 10 ml. for fat determination.

Where the formation of fat in absence of CO_2 was measured, the flasks were provided with a centre-well containing 0·5 ml. 5 N-NaOH and a roll of filter paper to absorb the CO_2.

Under the conditions employed no growth of the yeast occurs in the experimental period, as shown by the cell count (see Table 6).

Terms used (conversion coefficient). In experiments on fat formation, the fat formed is expressed in mg. C of fat formed in 10 ml. of yeast suspension, i.e. in about 50 mg. yeast. The utilization of substrate is expressed in mg. substrate used in 10 ml. suspension. The term *conversion coefficient*, i.e. the percentage of C of substrate converted into C of fat, is used as a measure of the efficiency of the conversion process. For glucose as substrate the conversion coefficient is represented by the expression:

$$\frac{\text{mg. C of fat formed} \times 100}{\text{mg. of glucose used} \times 40} \times 100\,\%.$$

The conversion coefficient is directly related to the R.Q., i.e. the R.Q. increases above 1·0 with increasing conversion coefficient.

Because of the experimental error involved in the estimation of glucose and the C of fat, only differences in the conversion coefficient of more than 2% are significant.

On the assumption that in the course of fat synthesis glucose is first broken down to 2 mol. of C_2 compounds and 2 mol. of CO_2, and that the C_2 compounds are then condensed to fatty acids, the highest conversion coefficient theoretically possible is 66·7 % (Magnus-Levy, 1925). Since *T. lipofera* metabolizes glucose only aerobically, a loss of C in the course of fat formation higher than the above may be expected and the maximal conversion coefficient therefore lower. Terroine & Bonnet (1927), measuring the caloric value of glucose used and fat formed in *Sterigmatocystis nigra*, arrived at a proportion of 4·38 mol. of glucose used per 1 mol. of fatty acid formed (oleic acid); the conversion coefficient according to these experiments would be 71 %. by the constant conversion coefficient. A cell count, carried out at the beginning and at the end of the experimental period, showed that no cell multiplication had taken place. In absence of substrate there was a disappearance of fat, linear with time.

The conversion coefficient found at 25° in the presence of $PO_4^{\equiv}$ (see below) varied between 11 and 30 %, usually between 18 and 25 %. A decrease in the conversion coefficient was observed when the washed yeast suspension was kept in the ice-chest for more than 24 hr.

The observation of Lodder (1934), that *T. lipofera* does not ferment glucose is confirmed. Under anaerobic conditions no CO_2 is formed from glucose by the yeast, and no glucose is used.

Table 5. *Oxygen uptake*, CO_2 *output and glucose utilization in* T. lipofera

(26·2 mg. yeast (dry wt.), 0·1 M-phosphate buffer pH 5·5; 25°; 3 hr.)

Substrate	Oxygen used (μl.)	CO_2 output (μl.)	Glucose used (μl.)	R.Q.	$\frac{\mu l.\ O_2\ used}{\mu l.\ glucose\ used}$
0	262	202	—	0·77	—
Glucose	1988	2990	1130	1·50	1·76

Table 6. *Time curve of fat formation from glucose in* T. lipofera

(49·4 mg. yeast (dry wt.)/10 ml.; 0·1 M-phosphate buffer pH 6·7; 120 mg. glucose/10 ml.; 25°.)

	Substrate					
	None			Glucose		
Time (hr.)	Fat found (mg. C)	Fat used (mg. C)	Glucose used (mg.)	Fat found (mg. C)	Fat formed (mg. C)	Conversion coefficient (%)
0	8·69	—	—	—	—	—
3	8·50	0·19	21·6	9·65	0·96	11·1
6	8·35	0·34	50·8	10·93	2·25	11·1
9	7·90	0·80	71·5	12·40	3·71	12·9

Cell count (in presence of glucose): initial, 316×10^6 cells/ml.; after 9 hr., 307×10^6 cells/ml.

RESULTS

Oxidation of glucose and formation of fat. Glucose is rapidly oxidized by *T. lipofera*. The Q_{O_2} in presence of glucose varied between 20 and 30, 1·3–2·0 mol. O_2/mol. glucose being used. Since 6 mol. of O_2 are required for the complete combustion of 1 mol. of glucose, the oxidation of glucose by this organism is incomplete. The R.Q. found was always higher than 1·0, varying between 1·15 and 1·5, and indicating a formation of fat from glucose. In the absence of substrate the Q_{O_2} was 3–7, the R.Q. varying from 0·7 to 0·95, indicating an oxidation of stored fat (Table 5).

Table 6 shows that glucose utilization and fat formation increase linearly with time. In the recorded experiment the fat content increased in the course of 9 hr. from 8·69 to 12·4 mg. C/49·4 mg. yeast, i.e. from 23 to 33 %. The C of fat formed remained proportional to the glucose used as shown

Effect of pH *on* R.Q. *and fat formation.* The effect of pH between 5·5–7·5 on the R.Q. and fat formation (Table 7) shows that with increasing pH the O_2 uptake and the R.Q. decrease. At pH 7·5 the R.Q. was 0·74; in other experiments the R.Q. at that pH was about 1·0. As indicated by the measurements of R.Q., the fat formation decreases with increasing pH and was found highest at pH 5·5–6·0 (Fig. 4). The conversion coefficient was also highest at pH 5·5.

Effect of temperature on fat formation. The effect of temperature on fat formation was studied by shaking simultaneously a number of flasks in baths of varying temperatures (15–37°). The utilization of glucose reached a peak at 25–30°, the fat formation was highest at 25°. The conversion of carbohydrate into fat was highest at 20–25°, i.e. at the optimal growth temperature of the organism (Table 8).

Effect of oxygen and CO_2. The effect of oxygen was examined because, under our experimental

Table 7. *Effect of* pH *on* R.Q.

(14·0 mg. cells (dry wt.); 0·1 M-phosphate buffer, glucose; 25°, 3 hr.)

	pH			
	5·5	6·5	7·0	7·5
O_2 uptake (μl.)	1203	867	555	400
CO_2 output (μl.)	1448	970	637	297
R.Q.	1·20	1·18	1·15	0·74

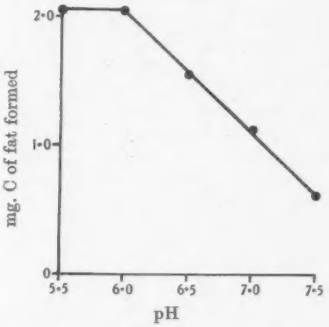


Fig. 4. Effect of pH on fat formation. 53·5 mg. yeast (dry wt.); 0·1 M-phosphate buffer; 100 mg. glucose/10 ml.; 25°; 3 hr.

Table 8. *Effect of temperature on fat formation*

(50·5 mg. yeast (dry wt.); 0·1 M-phosphate buffer pH 5·5; 80 mg. glucose/10 ml.; 5 hr.)

	Temperature				
	15°	20°	25°	30°	37°
Glucose used (mg.)	16·1	25·6	38·7	35·3	12·9
C content of fat formed (mg.)	0·21	2·29	3·05	2·77	0·30
Conversion coefficient (%)	3·1	22·3	20·1	19·7	5·8

conditions as compared with the conditions for optimal growth, it might be a limiting factor in fat formation. The effect of CO_2 was studied in view of the known role of CO_2 in biological synthesis

Table 9. *Effect of oxygen and* CO_2 *on fat formation*

(50·5 mg. yeast (dry wt.); 0·1 M-phosphate buffer pH 6·0; 100 mg. glucose/10 ml.; 25°; 6 hr.)

Gas mixture	Glucose used (mg.)	C of fat formed (mg.)	Conversion coefficient (%)
Air; NaOH	58·3	5·81	24·9
Air + 5% CO_2	51·3	4·83	23·6
50% O_2; NaOH	59·5	6·21	26·1
50% O_2 + 5% CO_2	49·2	4·83	24·5

(Stephenson & Krebs, 1941). The results recorded in Table 9 show that increased concentration of oxygen has no appreciable effect on the utilization of glucose or on the conversion of carbohydrate to fat. The presence of CO_2 decreases the utilization of carbohydrate and the fat formation, but does not affect the conversion coefficient.

Effect of NH_4^+ *on respiration and fat formation.* It is known from work on *Endomycopsis vernalis* and other yeasts that the formation of fat from carbohydrate is impaired in the presence of nitrogenous substances owing to the use of carbohydrate for growth (Nägeli & Loew, 1878; Smedley MacLean, 1922; Raaf, 1941). The effect of NH_4Cl on the respiration and the fat formation was therefore examined. Since the lag period of the growth of *T. lipofera* was found to be about 6 hr., no appreciable multiplication of the cells could have taken place during the experimental period (3–4 hr.).

Table 10 shows that increasing concentrations of NH_4Cl (0·067–0·2 M) decrease the oxidation of glucose. The formation of fat and the conversion coefficient are decreased by increasing concentrations of NH_4Cl (0·025–0·2 M) (Table 11). Urea, which has been shown by Lodder (1934) to serve as a source of N for the growth of the organism, has an effect similar to that of NH_4Cl on the formation of fat and the conversion coefficient (Table 12).

Table 10. *Effect of* NH_4Cl *on the respiration of* T. lipofera

(26·5 mg. yeast (dry wt.); 0·1 M-phosphate buffer pH 5·5; 15 mg. glucose; 25°; 3 hr.)

NH_4Cl concentration (M)	O_2 uptake (μl.)	Glucose used (mg.)
0	1871	10·76
0·067	1797	9·58
0·134	1600	8·53
0·200	1450	7·80

Table 11. *Effect of* NH_4Cl *on fat formation*

(50·2 mg. yeast (dry wt.)/10 ml.; 0·1 M-phosphate buffer pH 6·0; 50 mg. glucose/10 ml.; 25°; 4 hr.)

NH_4Cl concentration (M)	Glucose used (mg.)	C of fat formed (mg.)	Conversion coefficient (%)
0	44·2	3·14	17·7
0·025	43·5	2·89	16·5
0·05	41·8	2·20	13·1
0·20	35·8	1·39	9·7

Table 12. *Effect of urea on fat formation*

(52·0 mg. yeast (dry wt.)/10 ml.; 0·1 M-phosphate buffer pH 6·0; 100 mg. glucose/10 ml.; 25°; 4 hr.)

Urea concentration (M)	Glucose used (mg.)	C of fat formed (mg.)	Conversion coefficient (%)
0	31·9	2·74	21·4
0·01	30·5	2·78	22·8
0·03	30·1	2·29	19·0
0·10	31·0	1·82	14·6

Effect of $PO_4^{\equiv}$ *on fat formation.* Smedley MacLean & Hoffert (1923, 1924) were the first to show that the phosphate ion is of importance for the conversion of carbohydrate into fat. As shown in Table 13, $PO_4^{\equiv}$ has no effect on the utilization of glucose, but considerably enhances the conversion of carbohydrate into fat. Under the experimental conditions used, 0·001 M-KH_2PO_4 was sufficient to raise the conversion coefficient to the maximum; higher concentrations of $PO_4^{\equiv}$ had no further effect. The experiments confirm the importance of $PO_4^{\equiv}$ in the synthesis of fat from carbohydrate. The formation of some fat in absence of added $PO_4^{\equiv}$ is no doubt due to the phosphate present in the cell.

Table 13. *Effect of* $PO_4^{\equiv}$ *on fat formation*

(0·1 M-phthalate buffer pH 6·0; 100 mg. glucose/10 ml.; 25°; 5 hr.)

KH_2PO_4 concentration (M)	Glucose used (mg.)	C of fat formed (mg.)	Conversion coefficient (%)
Exp. 1, 52·8 mg. yeast:			
0	53·9	2·10	9·8
0·001	51·0	3·87	19·5
0·01	50·6	3·79	18·8
0·05	51·3	3·99	19·5
Exp. 2, 50·0 mg. yeast:			
0	41·7	2·52	15·1
0·001	40·1	3·16	19·6

Effect of Mg^{++} *and* Mn^{++} *etc., on fat formation.* Ohlmeyer & Ochoa (1937) have shown that Mg^{++} is of importance in the carbohydrate breakdown in yeast, and that it can be replaced by Mn^{++}. The effect of additions of Mg^{++} (0·01–0·1 M) or Mn^{++} (0·001–0·01 M) on the formation of fat from glucose by *T. lipofera* was therefore examined. The experiment recorded in Table 14 shows that additional Mg^{++} or Mn^{++} has no effect on the conversion of carbohydrate to fat. This result does not exclude the possible role of Mg, since enough Mg for the metabolism of the organism may be present in its cells.

Table 14. *Effect of* Mg^{++}, Mn^{++}, *aneurin and choline on fat formation*

(49·6 mg. yeast (dry wt.)/10 ml.; 100 mg. glucose/10 ml.; 0·1 M-phosphate buffer pH 6·0; 25°; 6 hr.)

Addition	Glucose used (mg.)	C of fat formed (mg.)	Conversion coefficient (%)
0	46·0	4·49	24·5
0·01 M-$MgSO_4$	44·0	4·32	24·6
0·1 M-$MgSO_4$	45·0	4·46	24·8
0·001 M-$MnSO_4$	43·7	4·54	26·0
0·01 M-$MnSO_4$	42·8	4·35	25·4
Aneurin 1 μg./ml.	44·5	4·62	26·0
Choline 1 mg./ml.	47·5	4·70	24·8

Additions of aneurin or choline chloride had no effect on the fat formation from glucose, though these two substances have been shown to be of importance in the fat metabolism of the animal (McHenry & Gavin, 1941; Perlmann & Chaikoff, 1939).

Effect of iodoacetate on respiration and fat formation. The effect of iodoacetate, which is known as an inhibitor of carbohydrate breakdown, on the oxidation of glucose and formation of fat was examined as it was thought that it might shed some light on the intermediate carbohydrate breakdown products formed in the course of fat synthesis. Table 15 shows that, in absence of substrate, 0·0003 M-iodoacetate has little effect on the respiration or R.Q. of *T. lipofera*. In the presence of glucose iodoacetate considerably inhibits the oxygen uptake and reduces the R.Q. to that of the control without substrate, i.e. below 1·0. A similar effect of iodoacetate, in reducing the R.Q. of frog muscle below 1·0, has been described by Meyerhof & Boyland (1931). It should be noted that whilst in baker's yeast iodoacetate does not inhibit the oxidation of glucose (Lundsgaard, 1930), in *T. lipofera* the oxygen uptake in the presence of glucose is considerably inhibited. Bernheim (1942) also found an inhibition of the oxidation of glucose by iodoacetate in the aerobic yeast *Blastomyces dermatiditis*.

Table 15. *Effect of iodoacetate on the respiration of* T. lipofera

(20·7 mg. yeast; 0·1 M-KH_2PO_4; 25°; 2 hr.)

Substrate	Iodoacetate	O_2 uptake (μl.)	CO_2 output (μl.)	R.Q.
0	0	284	273·5	0·96
0	0·0003 M	242	225·5	0·93
0·01 M-glucose	0	945	1078	1·14
0·01 M-glucose	0·0003 M	324	306	0·94

Table 16. *Effect of iodoacetate on fat formation*

(Exps. 1 and 2: 0·1 M-phthalate buffer pH 6·0; 0·001 M-KH_2PO_4. Exp. 3: 0·1 M-phosphate buffer pH 5·5; 100 mg. glucose/10 ml.; 25°; 5 hr.)

Exp. no.	Iodoacetate concentration (M)	Glucose used (mg.)	C of fat formed (mg.)	Conversion coefficient (%)
(1) 52·8 mg. yeast	0	50·6	+3·79	18·8
	0·0002	43·6	+3·23	18·5
(2) 50·0 mg. yeast	0	—	−0·03	—
	0	40·1	+3·16	19·65
	0·0005	—	−0·18	—
	0·0005	13·0	−0·05	—
(3) 50·0 mg. yeast	0	43·8	+2·36	13·5
	0·0005	18·4	−0·58	—
	0·00025	30·6	−0·10	—
	0·00017	34·8	+0·98	7·0

As shown in Table 16, iodoacetate inhibits the fat synthesis. At concentrations higher than

0·00025M some glucose is utilized, accompanied by a disappearance of fat; this corroborates the results obtained by R.Q. measurements. Concentrations of iodoacetate below 0·00025M inhibit to some extent both glucose utilization and fat synthesis. Some fat is formed in the presence of such low concentrations of iodoacetate, and, as shown in Exp. 1 (Table 16), the conversion coefficient is in some cases identical with that of the control without iodoacetate. These experiments suggest that the action of iodoacetate is due primarily to a specific inhibition of the carbohydrate breakdown, though it does not appear to influence the synthesis of fat from carbohydrate breakdown products.

Distribution of acid-soluble P *in* T. lipofera *in the presence of iodoacetate and fluoride*

Macfarlane (1936) has shown that considerable changes occur in the distribution of P in the yeast cell during fermentation and that the pyrophosphate-P and organic-P fractions increase in the presence of iodoacetate or fluoride. Since it was shown above that in the presence of 0·0003–0·0005M-iodoacetate *T. lipofera* does not form fat, but can utilize some glucose, the changes of P distribution in this yeast during the oxidation of glucose in the presence of iodoacetate and fluoride were studied.

Equal amounts of a suspension of freshly collected yeast (grown in liquid medium) in 0·1M-NaCl containing 0·002M-KH_2PO_4 were placed into three 1 l. cylinders (1·725 g. yeast (dry wt.)/cylinder). The contents of one cylinder were immediately centrifuged off, the yeast cake washed with saline and then twice extracted with 50 ml. 4% trichloroacetic acid. To the remaining two cylinders iodoacetate (final concentration 0·0003M) and sodium fluoride (0·025M) respectively were added; after 10 min. glucose was added and the contents of the cylinders were well aerated for 6 hr. at room temperature (18°) by passing compressed air through porous blocks. Details of the contents of the cylinders are given in the legend of Table 17. At the end of the experimental period the contents of the cylinders were treated as described for the control above. Samples of the trichloroacetic acid extracts were then taken for estimations of the various P fractions as described by Macfarlane (1939).

The results (Table 17) show that in the presence of both iodoacetate and sodium fluoride there is a disappearance of orthophosphate-P, no appreciable change in the pyrophosphate-P, and a considerable increase in the organic-P. The total acid-soluble P was also increased. The increase of the organic-P in the presence of iodoacetate (1·12 mg. P/1 g. yeast) is nearly accounted for by the increase in hexosediphosphate (0·92 mg. P, i.e. 82% of the organic-P formed). It should be noted that under the experimental conditions described the alkali-labile P (triosephosphate-P) decreased. Iodoacetate is, under anaerobic conditions, or in simple enzymic systems, regarded as a specific inhibitor of the triosephosphate dehydrogenase, and thus brings about an accumulation of alkali-labile-P in the course of carbohydrate breakdown; the present result, therefore, if confirmed by isolation of hexosediphosphate, suggests some differences between oxidative and anaerobic phosphorylation in yeast cells (cf. Mann, 1943). Sodium fluoride brings about an increase of organic phosphate, which is only to a small extent hydrolyzable by N-HCl at 100° in 180 min. If there exists a parallelism between the anaerobic carbohydrate breakdown in other yeasts, and the aerobic phosphorylation in *T. lipofera*, this result would suggest a formation of phosphoglyceric acid.

Carbon balance sheet in T. lipofera

The experiments were carried out in the following way (see Birkinshaw & Raistrick, 1931):

A suspension of yeast (2·5–3·1 g. dry wt./100 ml.) in 0·1M-NaCl containing glucose and 0·01–0·05M-KH_2PO_4 was prepared. One hundred ml. of this suspension were immediately spun off, the yeast cake washed once with 0·1M-NaCl and once with water, and the yeast freeze-dried to constant weight over P_2O_5. The supernatant liquor and the washings were combined and samples of the solution used for the estimation of the various compounds as shown below. One hundred ml. of the yeast suspension were placed in a 3 l. conical flask and CO_2-free air passed at a rate of approximately 100 ml./min. through this suspension whilst it was shaking in a thermostat at 25°. The air passing out of the flask was first dried over $CaCl_2$, and then passed through a potash bulb and three soda-lime U-tubes followed by one of $CaCl_2$. A baryta trap following the U-tubes showed that in this arrangement all the CO_2 was absorbed. After the experimental period (5 hr.) the yeast was spun off and treated in the same way as described above.

In the freeze-dried yeast the C, N, fat and carbohydrate contents were estimated: C according to Van Slyke & Folch

Table 17. P *distribution in* T. lipofera

(Each cylinder contains 450 ml. 0·1M-NaCl and 0·002M-KH_2PO_4, with the following additions:
(1) 1·725 g. yeast.
(2) 1·725 g. yeast; 0·0003M-iodoacetate; 5 g. glucose.
(3) 1·725 g. yeast; 0·025M-NaF; 5 g. glucose.)

	mg. acid-soluble P/g. yeast as					
Cylinder	Orthophosphate	Pyrophosphate	Organic-P	Hexose diphosphate	Alkali labile-P	Total
1	1·15	2·04	2·14	1·75	0·30	5·27
2	0·85	2·06	3·26	2·68	0·14	6·17
3	0·70	2·26	3·18	2·12	0·23	6·15

Table 21. *Formation of fat from various carbohydrates*

(50–52 mg. yeast (dry wt.); 0·1 M-phosphate buffer pH 5·5; 5 hr.; 25°; 100 mg. substrate/10 ml. Yeast in Exps. 1–3 grown on glucose; in Exp. 4 grown on sucrose.)

Exp.	Substrate	C of fat formed (mg.)
1	Glucose	5·80
	Fructose	5·50
	Sucrose	3·68
	Maltose	3·36
2	Glucose	2·33
	Galactose	0·43
3	Glucose	2·12
	Sucrose	1·38
4	Glucose	2·81
	Sucrose	2·96

claimed that acetaldehyde is converted into fat by *Endomycopsis vernalis*; Smedley MacLean & Hoffert (1926) were unable to demonstrate a synthesis of fat from acetaldehyde by baker's yeast.

In the presence of pyruvate, lactate, dihydroxyacetone, gluconate, butyrate, α-ketoglutarate and mixed amino-acids (tryptic casein digest) no fat was formed by *T. lipofera* under the experimental conditions employed. In contrast to this yeast, *Endomycopsis vernalis* and baker's yeast have been shown to form fat from pyruvate and lactate (Haehn & Kintoff, 1926; Smedley MacLean & Hoffert, 1926; Smythe, 1938).

DISCUSSION

The results reported above show that the aerobic yeast *T. lipofera* is capable of storing large amounts

Table 22. *Formation of fat in* T. lipofera *in presence of ethanol and acetic acid*

(Exp. 1: 50·6 mg. yeast/10 ml.; 0·1 M-phosphate buffer pH 6·7; 25°; 4 hr.
Exp. 2: 50·3 mg. yeast/10 ml.; 0·1 M-phosphate buffer pH 5·5; 25°; 5 hr.)

Exp.	Substrate	Glucose used (mg.)	C of fat formed (mg.)	Conversion coefficient (%)
1	0	—	−1·15	—
	0·06 M-glucose	20·2	+2·70	33·4
	0·1 M-ethanol	—	+0·38	—
	0·06 M-glucose + 0·1 M-ethanol	16·6	+1·70	25·6
	0·06 M-acetate	—	+0·02	—
	0·06 M-glucose + 0·06 M-acetate	11·8	+1·58	33·5
2	0·06 M-glucose	36·6	+2·07	14·1
	0·1 M-ethanol	—	−0·46	—
	0·06 M-glucose + 0·1 M-ethanol	31·3	+1·79	14·3
	0·1 M-acetate	—	−0·50	—
	0·06 M-glucose + 0·1 M-acetate	3·0	+0·26	(21·6)*

* On account of the small glucose uptake and fat formation the conversion coefficient is not reliable.

(Lindner, 1922; Stephenson & Whetham, 1922, 1923; Smedley MacLean & Hoffert, 1923, 1924, 1926; Haehn & Kintoff, 1926). The formation of fat from these substrates in absence and in presence of glucose by *T. lipofera* was therefore examined at pH 6·7 and 5·5. As shown in Table 22, no fat is formed from ethanol or acetic acid in absence of glucose, and both substrates inhibit to some extent the formation of fat from glucose. Since ethanol is rapidly oxidized by this organism, but is not converted into fat, this result appears to exclude ethanol as an intermediate in the fat synthesis. Acetic acid is only slowly oxidized; the failure to find fat formation from this substrate, however, cannot be considered to be conclusive evidence that it does not play any role as intermediate in the synthesis of fat. The permeability of the cell membrane to acetic acid may be a limiting factor in the metabolism of this yeast.

In the presence of acetaldehyde, small but definite amounts of fat were formed by *T. lipofera* (Table 23), although acetaldehyde is only very slowly oxidized by this yeast (Table 19). Haehn & Kintoff (1926)

Table 23. *Formation of fat from various substrates*

(50–52 mg. yeast/10 ml.; 0·1 M-phosphate buffer pH 5·5; 25°; 5 hr.)

Exp.	Substrate	C of fat formed (mg.)
1	0·06 M-glucose	+1·86
	0·05 M-gluconate	+0·26
	0·05 M-dihydroxyacetone	−0·19
	0·05 M-pyruvate	−1·24
	0·05 M-lactate	−0·13
2	0·06 M-glucose	+2·33
	0·05 M-gluconate	−0·71
	0·05 M-pyruvate	−1·69
3	0·06 M-glucose	+2·12
	0·02 M-acetaldehyde	+1·28
4	0·06 M-glucose	+1·69
	0·02 M-acetaldehyde (duplicate)	+0·25; +0·51
	Tryptic casein digest, 0·1 mg. N/ml. (duplicate)	−0·48; −0·49
5	0·06 M-glucose	+1·81
	0·04 M-butyrate (duplicate)	−0·06; −0·05
	0·02 M-acetaldehyde (duplicate)	+0·66; +0·60
	0·04 M-α-ketoglutarate	−0·16

of fat in its cells. The conversion of carbohydrate into fat by this organism appears to be more efficient than in *Endomycopsis vernalis*. No detailed information is available about *Nectaromyces reukaufii*, which has been recently shown capable of storing large amounts of fat (Rippel, 1943).

Under anaerobic conditions no glucose is utilized. The carbohydrate breakdown and fat formation in this yeast are therefore both aerobic processes. The fat formation is accompanied by an increase in the carbohydrate of the cell (Table 18). A number of authors have found that other yeasts, e.g. baker's yeast or *Endomycopsis vernalis*, form fat only under aerobic conditions; this observation has, however, not found a sufficient expression in the various schemes put forward for possible pathways of the synthesis of fatty acids from carbohydrate.

The investigation of the effect of additions of Mg^{++}, Mn^{++} and aneurin to the yeast suspension has not disclosed any part played by these compounds in the fat synthesis. Possibly the yeast cell contains sufficient amounts of these compounds to maintain its metabolic activities at a high rate even without further additions, provided sufficient carbohydrate substrate is present. Addition of phosphate appears, however, to be necessary for maximal fat synthesis from carbohydrate.

Acetaldehyde was the only non-carbohydrate substrate of a number tested, in the presence of which fat was formed. A similar observation in other yeasts, as well as that of fat formation in the presence of pyruvate, has hitherto been regarded as evidence that these substrates are intermediates in the synthesis of fat from carbohydrate. It will be noted that the yeast contains a considerable amount of carbohydrate, which might be mobilized in the presence of some substrates and used for synthesis of fat. The result obtained with acetaldehyde cannot, therefore, be considered as conclusive evidence that acetaldehyde is an intermediate in the synthesis of fat from carbohydrate. Such evidence might be provided by the use of acetaldehyde labelled with isotopic C, or by a further investigation of the metabolism of acetaldehyde in this yeast.

SUMMARY

1. The growth and metabolism of *Torulopsis lipofera*, a yeast rich in fat, have been investigated.

2. *T. lipofera* grows well on a medium containing 0·05% marmite, carbohydrate, ammonia and mineral salts, and on molasses containing K, NH_4 and Mg salts. The optimum temperature is 23–25°. The generation time under optimal conditions is $5\frac{1}{4}$–$5\frac{1}{2}$ hr.

3. The content of fatty material (fatty acids and unsaponifiable matter) in the dry yeast varied between 18·6–43%, the unsaponifiable fraction forming 6·8–8·8%.

4. Methods for the macro- and micro-estimation of fat and for the study of fat formation in yeast are described.

5. In the presence of glucose the R.Q. of the yeast is higher than 1·0. The fat content increases linearly with time and is proportional to the glucose used. The conversion coefficient, i.e. the percentage of C of glucose used converted into C of fat, varied between 11 and 33%. In the absence of substrate the R.Q. is lower than 1·0 and fat is utilized.

6. The carbohydrate breakdown and fat formation in *T. lipofera* are aerobic processes. Under anaerobic conditions no glucose is used.

7. The conditions of fat formation from glucose were studied. The fat formation is highest at pH 5·5–6·0; the optimal temperature is 20–25°; increasing concentrations of NH_4Cl and urea inhibit the conversion of carbohydrate into fat.

8. Phosphate is essential for maximal conversion of carbohydrate to fat.

9. Iodoacetate (0·0002–0·0005 M) inhibits the fat formation. In the presence of iodoacetate and glucose there is an increase in the organic-P, the hydrolysis curve of which indicates an accumulation of hexosediphosphate. In the presence of fluoride there is an accumulation of organic-P, which is only to a small extent hydrolyzable.

10. The metabolism of glucose in *T. lipofera* was studied by the C balance sheet method. 97% of the C used was accounted for. Glucose is partly oxidized to CO_2 and partly converted to fatty acids and cell-carbohydrate.

11. The oxidation of various substrates in *T. lipofera* was examined. Glucose, fructose, maltose, ethanol are rapidly oxidized. Saccharose, galactose, dihydroxyacetone, acetate, butyrate, methylglyoxal and stearate are oxidized more slowly, whilst pyruvate, lactate, acetaldehyde, propionate, glycerol, glyceraldehyde, succinate, fumarate, *dl*-malate, citrate and gluconate are little, if at all, oxidized. Oleate decreases the O_2 uptake as compared with the control without substrate.

12. The formation of fat from various non-carbohydrate substrates was studied, acetaldehyde alone of the substrates tested giving a significant increase in fat formation.

The author wishes to express his thanks to Dr M. Stephenson and Dr R. Hill for their advice and criticism in the course of this work, and also to Prof. A. C. Chibnall, F.R.S., for his encouragement. He is greatly indebted to Dr A. C. Thaysen and his staff, Chemical Research Laboratory, Teddington, for facilities to work in his laboratory and for help in growing the yeast on a semi-large scale. The author wishes also to thank Mr H. Mowl for his technical assistance. The work was made possible by a whole-time grant of the Medical Research Council.

REFERENCES

Belin, P. (1926). *Bull. Soc. Chim. biol., Paris*, **8**, 1081.
Bernheim, F. (1942). *J. Bact.* **44**, 533.
Birkinshaw, J. H. & Raistrick, H. (1931). *Philos. Trans.* B, **220**, 11.
Bloor, W. R. (1929). *J. biol. Chem.* **82**, 273.
den Dooren de Jongh, L. E. (1926). *Ned. Tijdschr. Hyg.* **1**, 136.
Fink, H., Haehn, H. & Hoerburger, W. (1937). *Chem. Ztg*, **61**, 689, 723, 744.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Friedemann, T. E. & Graeser, J. B. (1933). *J. biol. Chem.* **100**, 291.
—— & Klaas, R. (1936). *J. biol. Chem.* **115**, 47.
Haehn, H. & Kintoff, W. (1926). *Chem. Zelle*, **12**, 115.
Kleinzeller, A. & Trim, A. R. (1944). *Analyst*, **69**, 241.
Krebs, H. A., Smyth, D. H. & Evans, E. A. (1940). *Biochem. J.* **34**, 1041.
Kumagawa, M. & Suto, K. (1908). *Biochem. Z.* **8**, 212.
Lane, J. H. & Eynon, L. (1923). *J. Soc. chem. Ind., Lond.*, **42**, 32T.
Lemeland, P. (1923). *Bull. Soc. Chim. biol., Paris*, **5**, 110.
Lindner, P. (1922). *Z. angew. Chem.* **35**, 110.
Lodder, J. (1934). *Die anascosporogenen Hefen.* Amsterdam: N. V. Noord-Hollandsche Uitge vers maatschapij.
Longenecker, H. E. (1941). *Biol. Symposia*, **5**, 99.
Lundsgaard, E. (1930). *Biochem. Z.* **220**, 8.
Macfarlane, M. G. (1936). *Biochem. J.* **30**, 1369.
—— (1939). *Biochem. J.* **33**, 565.
McHenry, E. W. & Gavin, G. (1941). *J. biol. Chem.* **138**, 471.
Magnus-Levy, A. (1925). *Oppenheimers Handb. Biochem.* **8**, 461. Jena: Fischer.
Mann, T. (1943). *Nature, Lond.*, **151**, 619.
Meyerhof, O. & Boyland, E. (1931). *Biochem. Z.* **237**, 406.
Miller, B. F. & Van Slyke, D. D. (1936). *J. biol. Chem.* **114**, 583.
Nägeli, C. & Loew, O. (1878). *Liebigs Ann.* **193**, 322.
Ohlmeyer, P. & Ochoa, S. (1937). *Biochem. Z.* **293**, 338.
Perlmann, I. & Chaikoff, I. L. (1939). *J. biol. Chem.* **127**, 211.
Prescott, S. C. & Dunn, C. G. (1940). *Industrial Microbiology.* New York and London: MacGraw Hill, Inc.
Raaf, H. (1941). Cited in *Chem. Zbl.* (1942), **113**, I, 3217.
Reichel, L. & Reinmuth, W. (1938). *Biochem. Z.* **299**, 359.
Rippel, A. (1943). *Naturwissenschaften*, **31**, 248.
Rosenmund, K. W. & Kuhnhenn, W. (1923). *Z. Untersuch. Nahr.- u. Genussm.* **46**, 154.
Smedley MacLean, I. (1922). *Biochem. J.* **16**, 370.
—— & Hoffert, D. (1923). *Biochem. J.* **17**, 720.
—— —— (1924). *Biochem. J.* **18**, 1273.
—— —— (1926). *Biochem. J.* **20**, 343.
Smythe, C. V. (1938). *J. biol. Chem.* **126**, 635.
Stephenson, M. (1939). *Bacterial Metabolism.* London: Longmans, Green and Co.
—— & Krebs, H. A. (1941). *Ann. Rep. Prog. Chem.* **38**, 257.
—— & Whetham, M. D. (1922). *Proc. Roy. Soc.* B, **93**, 262.
—— —— (1923). *Proc. Roy. Soc.* B, **95**, 200.
Terroine, E. F. & Bonnet, R. (1927). *Bull. Soc. Chim. biol., Paris*, **9**, 588.
Thaysen, A. C. & Morris, M. (1943). *Nature, Lond.*, **152**, 526.
Tošić, J. & Krebs, H. A. (1943). *Biochem. J.* **37**, *Proc.* xv.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Warburg, O. & Yabusoe, M. (1924). *Biochem. Z.* **146**, 380.

The Microbiological Degradation of Steroids

2. OXIDATION OF CHOLESTEROL BY *PROACTINOMYCES* SPP.

BY G. E. TURFITT, *The Home Office Forensic Science Laboratory, Nottingham*

(*Received* 6 *September* 1944)

The identification has been recorded (Turfitt, 1944) of species of the genus *Proactinomyces* as the bacterial agencies concerned in the disappearance of cholesterol from soils. Although many soil samples have been examined, no evidence has yet been adduced that any other bacteria, moulds or actinomycetes are able to accomplish this sterol breakdown; the possibility of the existence of cholesterol-decomposing organisms which do not grow on ordinary solid nutrient media is still being investigated. This apparently unique characteristic of *Proactinomyces* is the more interesting in view of the taxonomic position of the organisms. Classification within the Order Actinomycetales involves a gradation through the unbranched *Mycobacterium* and *Corynebacterium* to the richly branching *Actinomyces*, the genus *Proactinomyces* constituting an intermediate transitional stage. Since bacterial cells are, with few exceptions (Sifferd & Anderson, 1936), entirely devoid of any sterols, whilst the latter are actually synthesized by the yeasts and mould fungi, it is remarkable that one small section of the Natural

Order serving as a connecting link between the two groups should apparently be specific in effecting the catabolism of sterols.

The wide distribution of *Proactinomyces* spp. in well-aerated, fertile soils is in accord with their strictly aerobic character, and the utilization of cholesterol by the organisms when grown in pure culture with cholesterol as sole carbon source must be attributed to oxidation processes. Chemical oxidation of the cholesterol molecule has yielded a variety of products, and has amply demonstrated the close interrelationship between the sterols, the sex hormones and the bile acids (Fieser, 1937). In addition to such changes, which involve oxidative disruption of the side-chain, usually simultaneously with certain slight nuclear changes, it has recently been shown that the C_7 position is particularly susceptible to oxidation under relatively mild conditions. Thus, Bergström & Wintersteiner (1941, 1942 *a*, *b*, *c*) have isolated 7-hydroxy- and 7-keto-derivatives after aeration of cholesterol in colloidal aqueous solution at 85°, whilst similar results have been obtained by Windaus, Bursian & Riemann (1941) by photo-oxidation of cholesterol. In all these oxidations the cholane ring system has remained intact, but opening of ring A is readily accomplished by the hypobromite or ozone oxidation of Δ^4-cholestenone first described by Windaus (1906) prior to the establishment of the present sterol structure. It is a reasonable assumption that the microbiological oxidation follows an analogous course in that the molecule is again attacked at one or more of these vulnerable points. The necessary energy and available carbon required for continued bacterial growth is then derived from relatively simple degradation products split off from the parent structure.

EXPERIMENTAL

Rate of cholesterol degradation in vitro *by* P. erythropolis *Gray & Thornton*

A characteristic feature of the growth of *Proactinomyces* spp. on cholesterol with NH_4NO_3 as nitrogen source is the acid reaction of the medium after 4–7 days. Substitution of $(NH_4)_2SO_4$ for NH_4NO_3 again leads to the production of acid, which may thus arise from the liberation of acidic ions due to preferential utilization of the NH_4^+ radical. Support is given to this view by the fact that if KNO_3 replaces NH_4NO_3 in the medium, the latter gradually becomes slightly alkaline. At the same time, this hypothesis does not preclude the possibility of the simultaneous production of a water-soluble organic acid, a factor which would account for the alkalinity due to liberated basic ions never becoming very marked.

With a view to determining the optimum conditions for cholesterol decomposition, the rate of disappearance of cholesterol from *P. erythropolis* cultures has been investigated using three different media of known composition:

(*a*) Medium I: NH_4NO_3 0·1%; K_2HPO_4 0·025%; $MgSO_4.7H_2O$ 0·025%; NaCl 0·0005%; $FeSO_4.7H_2O$ 0·00001% in water.

(*b*) Medium II: medium I + 0·5% $CaCO_3$.

(*c*) Medium III: as medium I but with KNO_3 instead of NH_4NO_3.

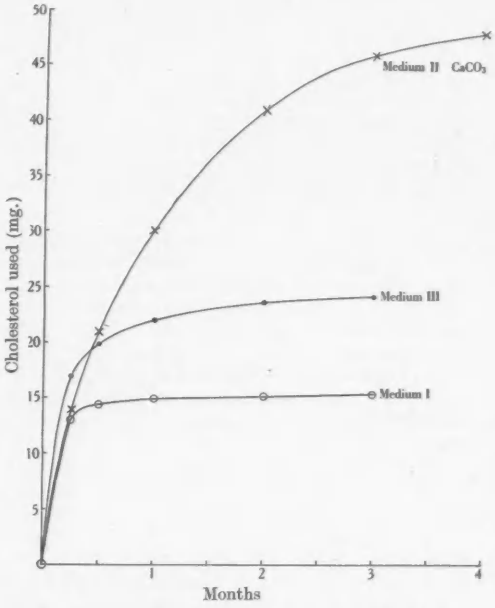


Fig. 1. Destruction of cholesterol by *P. erythropolis* cultures on synthetic media. For composition of media, see text. Temperature 25°.

Thirty-two 250 ml. conical culture flasks, each containing 100 ml. of the appropriate mineral salt solution, were autoclaved for 15 min. at 115°. 50 mg. finely powdered sterile cholesterol were added with aseptic precautions to each of the flasks, which were then inoculated with 0·5 ml. quantities of a suspension of *P. erythropolis* containing approx. 20 million viable organisms per ml. Sixteen of these flasks were incubated at 25° in darkness, whilst the remaining sixteen were maintained at the same temperature in diffused light. Throughout the incubation period the flasks were gently shaken every 24 hr. At definite intervals, two flasks (duplicates) from each batch of sixteen were removed. These cultures were extracted thoroughly with $CHCl_3$, the extracts from each culture dried with anhydrous Na_2SO_4 and made up to 100 ml. in a graduated flask.

The colorimetric determination of residual cholesterol in these solutions was carried out by a method essentially similar to that previously described (Turfitt, 1943), but

with slight differences in technique which considerably reduce the tedious nature of the analysis. A measured portion of each solution, containing approx. 0·5 mg. of cholesterol, was evaporated to dryness in a 15 ml. centrifuge tube, and the residue taken up in 1 ml. ether. 4 ml. of a 0·2% solution of digitonin in 95% ethanol were added, and the mixture concentrated to 3 ml. After standing overnight in the refrigerator, the white flocculent deposit was centrifuged off and washed three times with cold ether-acetone (1:2). 7 ml. benzene were added, and the solution was refluxed gently for 45 min. to decompose the digitonide, and then concentrated to 3 ml. Digitonin was precipitated by the addition of 9 ml. petroleum, b.p. 40–50°, centrifuged off after standing for 10 min., and washed three times with petroleum. The solution and petroleum washings were pooled, evaporated to dryness, and taken up in 5 ml. chloroform in a 10 ml. glass-stoppered cylinder. 0·5 mg. cholesterol dissolved in 5 ml. chloroform was measured into a second cylinder, and 1·0 ml. acetic anhydride and 0·1 ml. conc. H_2SO_4 added to each. After thorough mixing, each solution was allowed to stand in a bath at 25° for 15 min., the green colours being then compared in a colorimeter.

The results of these experiments are shown graphically in Fig. 1. No appreciable difference was observed between the flasks incubated in darkness and the corresponding ones exposed to light. The duplicate flasks examined at each time interval gave very closely concordant results.

In the unbuffered Media I and III the pH change occurring towards the seventh day brings about a marked decline in the degradation process, this decline being rather more evident where the change is a lowering of the pH value. In the presence of $CaCO_3$ to counteract acidity (Medium II), however, this relatively abrupt break in the curve is not experienced. The importance of the pH factor is thus experimentally established, and this, in conjunction with the obligatory aerobic nature of the organisms, provides a clear explanation of their relative rarity in acid peaty soils, and their abundance in aerated, lime-treated arable land.

Oxidation of cholesterol by Proactinomyces *spp.*

Since provision for the neutralization of acids was essential for any continued attack on the cholesterol molecule, the medium employed in an attempt to identify the reaction products had evidently to be buffered in some manner. The $CaCO_3$ used in the above work possessed obvious advantages, but it suffered from the inherent defect that it introduced a further source of carbon. Thus, before its use could be justified it was necessary to ascertain whether *Proactinomyces* spp. could utilize this inorganic carbon. In a series of carefully controlled experiments this point was investigated, but no evidence of any autotrophic character was detected.

For the preliminary large-scale work, a hundred 250 ml. conical culture flasks, each containing 100 ml. Medium I and approx. 1 g. $CaCO_3$, were autoclaved for 15 min. at 115°, and to each flask was added approx. 0·5 g. finely powdered sterile cholesterol. After inoculation with a heavy aqueous suspension of *P. erythropolis* prepared from a 24 hr. agar slope culture, the flasks were incubated in darkness at 25° for 1 month. At the end of this time the cultures were filtered at the pump on 18·5 cm. Whatman no. 5 filters, and the filtrate shaken mechanically with three successive 1 l. quantities of ether to remove neutral fat. The bulk of aqueous liquid was then adjusted to pH 4 with dilute H_2SO_4, and again thoroughly extracted with 1 l. quantities of ether. On evaporating this extract, after freeing from acid and drying with anhydrous Na_2SO_4, there were obtained 82 mg. of a yellowish oil (A) with a sharp, 'fatty acid' odour.

The residue from filtration of the cultures was dried at 60°, powdered, and extracted in a Soxhlet with ethanol for 48 hr. After this extraction there remained a mass consisting largely of $CaCO_3$, and of bacterial debris. Since this mass should also contain any insoluble Ca salts of organic acids produced during the bacterial action, it was accordingly acidified with dilute H_2SO_4 and the turbid suspension extracted continuously with ether for 8 hr. The washed and dried ether extract gave, on evaporation, 137 mg. of a brownish gum (B) from which a white amorphous solid tended to separate. The yellow ethanol solution from the Soxhlet extraction was separated by means of Girard's 'T' reagent into ketonic and non-ketonic fractions. The latter gave 21·4 g. of unchanged cholesterol, whilst the ketonic fraction, decolorized with charcoal and recrystallized twice from methanol-acetone (3:2), yielded 22·7 g. white needles, m.p. 80°. In a mixed m.p. determination with an authentic specimen of Δ^4-cholestenone the m.p. was not depressed. The compound was further characterized by means of the readily crystallized 2:4-dinitrophenylhydrazone. This derivative was obtained, after twice crystallizing from ethanol-benzene (1:1) as a mass of fine red rosettes, gradually changing to stout transparent prisms on standing, m.p. 233–234°, not depressed in admixture with an authentic specimen of Δ^4-cholestenone-2:4-dinitrophenylhydrazone. Found: N, 10·2%. Calc. for $C_{33}H_{48}O_4N_4$: N, 10·0%.

The initial stage of the bacterial attack on cholesterol is thus an oxidation of the —OH at C_3, accompanied by a rearrangement of the double bond to the 4:5 position. This formation of Δ^4-cholestenone is considered to result from the action of a dehydrogenase system in the cells of the inoculum; it represents merely a transfer of an atom of H, and does not involve the loss of either nuclear or side-chain carbon. Since bacterial growth continues actively, there must be a secondary action whereby either Δ^4-cholestenone is itself decomposed, or less probably, some unchanged cholesterol is oxidized in an alternative manner which yields a source of available carbon. In either event, the multiplying bacterial cells provide for further enzymic oxidation to Δ^4-cholestenone. Thus, in the earlier stages of the action it is this ketone which is the predominating product in the cultures. The small fractions (A) and (B) undoubtedly contain actual degradation products of the original sterol, but in this preliminary work the quantities available were insufficient to

permit of any serious attempt to isolate individual chemical entities.

The choice of *P. erythropolis* for this work was decided by the fact that it is by far the most frequently encountered species in the isolation of these cholesterol-decomposing organisms from soils. However, the activity of all the known *Proactinomyces* spp. (cultures of which have been obtained from the National Collection of Type Cultures, Lister Institute) has been investigated in a similar manner. Each organism was inoculated into a 1 l. conical culture flask containing 250 ml. Medium I, together with 1 g. $CaCO_3$ and 2 g. cholesterol, and the cultures incubated at 25° for 1 month. Extraction of the cultures as outlined above yielded Δ^4-cholestenone in every instance, although there was a wide variation in the yield obtained with the various species.

Isolation of Δ^4-cholestenone from soil treated with cholesterol

The oxidation of cholesterol to Δ^4-cholestenone by *Proactinomyces* spp. when grown in pure culture as described above occurs under conditions vastly different from those obtaining *in situ*, where the soil abounds with alternative carbon sources. In order to determine whether the oxidation follows the same course under natural conditions, 1·0 g. finely divided cholesterol was intimately mixed with 100 g. of a medium loam in a 250 ml. beaker, and buried below the ground surface in the soil from which the sample was taken. After 1 month, the experimental soil was removed from the ground, dried in an air-oven at 60°, and powdered in a mortar. By continuous extraction with acetone there were obtained 3·2 g. of a brownish gummy residue which yielded 0·85 g. of unsaponifiable matter. The ketonic fraction, separated by means of Girard's 'T' reagent, was a thick, yellow oil (0·217 g.), gradually setting to a solid mass on cooling in ice. Repeated crystallization from methanol-acetone (3:2) gave almost colourless needles, m.p. 78–79°, not depressed in admixture with Δ^4-cholestenone. The 2:4-dinitrophenylhydrazone crystallized from ethanol-benzene (1:1) in red rosettes, m.p. and mixed m.p. 233–234°. Found: N, 10·2%. Calc. for $C_{33}H_{48}O_4N_4$: N, 10·0%.

Oxidation of cholesterol by Proactinomyces *spp. in ordinary culture media*

In the experiments hitherto reported on microbiological oxidation in the sterol-sex hormone field, the steroid side-chain appears to exert a completely inhibitive influence on the action. Thus, Mamoli (1938) has described a bacterial mixture which, when cultured on Milan yeast, has smoothly converted dehydroandrosterone into androstenedione, and 3-pregnenol-20-one into progesterone; but cholesterol was completely unchanged. These results have been confirmed, and other steroid derivatives investigated, by Ercoli (1940, 1941) using *Micrococcus dehydrogenans* cultured on a buffered yeast extract.

Since *P. erythropolis* readily converts cholesterol into Δ^4-cholestenone in soil cultures, it was considered likely that the oxidation would also result in ordinary liquid culture media. Lemco broth and yeast water, both adjusted to pH 7·6, were selected for the experiment, and 100 ml. quantities of these were placed in 250 ml. conical culture flasks. The flasks were autoclaved for 15 min. at 115°, and 1 g. sterile powdered cholesterol was introduced into each. After inoculation with *P. erythropolis*, the flasks were incubated at 25° for 1 month. Extraction of the cultures with ether after this period gave, in both instances, an almost colourless crystalline mass. Separation of the ketonic fraction by means of the Girard 'T' reagent yielded 0·17 and 0·20 g. of slightly yellow crystalline solid from the broth and yeast water cultures respectively. Δ^4-Cholestenone was isolated in each case by crystallization of the ketonic residues from methanol-acetone (3:2), and characterized as its 2:4-dinitrophenylhydrazone.

DISCUSSION

Whilst no detailed results are yet available of the action of *P. erythropolis* on other steroid compounds, the oxidizing action of this organism is evidently not influenced by the presence of the side-chain to an extent comparable with that reported for *M. dehydrogenans*. These facts are of interest in connexion with the fate of ingested cholesterol. The former view that coprosterol originates by direct bacterial hydrogenation of cholesterol in the alimentary tract has received no experimental support, and it is now considered (Rosenheim & Starling, 1933; Rosenheim & Webster, 1943*a*, *b*) that the conversion occurs in two stages, Δ^4-cholestenone being the actual precursor of coprosterol. The possibility of the intestinal flora and fauna participating in the process has been closely investigated by these workers, who conclude that bacterial and protozoal agencies are probably not involved in either the oxidation or reduction stages. Although the discovery of the oxidation of cholesterol by *Proactinomyces* spp. appears to raise some superficial objections to this conclusion, it is doubtful whether any appreciable quantity of Δ^4-cholestenone arises in this manner *in vivo*. The author, in some as yet uncompleted experiments on this subject, has isolated *P. erythropolis* from lettuce, strawberries and other uncooked, edible produce, so that a number of these organisms may be assumed to be ingested with certain foodstuffs. The number of such organisms, however, must be relatively small, and will certainly be

further reduced by the highly acid conditions prevailing in the stomach. Any oxidation which these bacteria may accomplish in the alimentary canal will probably occur during passage through the small intestine, but it is extremely doubtful whether in this limited time more than a small percentage of the sterol would be oxidized. All attempts to recover the organisms from faeces have proved unsuccessful. In the case of the guinea-pig, the caecum has recently been identified (Schmidt & Hughes, 1942) as a principal site of the catabolism of cholic acid, the —OH groups of which are oxidized with formation of the corresponding keto-compounds. This oxidation is of a bacterial nature, as shown by the ability of *Alcaligenes faecalis* Castellani & Chalmers, isolated from the caecal contents, to effect the same oxidations *in vitro* (Schmidt, Hughes, Green & Cooper, 1942). The organism, cultured in serum, also oxidizes lithocholic acid and dehydro*iso*-androsterone to the corresponding keto-compounds (Hughes & Schmidt, 1942). In the author's experiments, neither *A. faecalis* nor the mixed flora of the human caecum has brought about the oxidation of cholesterol. The question, however, whether Δ^4-cholestenone production *in vivo* is essentially a bacterial oxidation, or whether it results from the enzymic activity of alimentary secretions, still cannot be regarded as settled.

SUMMARY

1. Cholesterol degradation by *Proactinomyces erythropolis* Gray & Thornton *in vitro* was greatly influenced by pH changes in the medium. In buffered media, disappearance of cholesterol was almost complete in 4 months.

2. All *Proactinomyces* spp. tested experimentally oxidized cholesterol to Δ^4-cholestenone in a varying degree when grown in mineral salt solution with the sterol as sole organic carbon source. It is concluded that oxidation of some of the sterol is effected by the cells of the inoculum, and that actual degradation of the molecule then occurs to provide available carbon for continued bacterial growth. Further oxidation to Δ^4-cholestenone then occurs, and the process continues until the whole of the cholesterol has disappeared. Certain fractions representing degradation products have been isolated in large-scale experiments, but not as yet identified.

3. Oxidation to Δ^4-cholestenone also resulted when cholesterol intimately mixed with garden soil was buried in the ground for 1 month.

4. In spite of evidence that *Proactinomyces* spp. are taken into the alimentary tract with certain foodstuffs, it is considered unlikely that these organisms play any appreciable role in the formation of Δ^4-cholestenone *in vivo*.

REFERENCES

Bergström, S. & Wintersteiner, O. (1941). *J. biol. Chem.* **141**, 597.
—— —— (1942*a*). *J. biol. Chem.* **143**, 503.
—— —— (1942*b*). *J. biol. Chem.* **145**, 309.
—— —— (1942*c*). *J. biol. Chem.* **145**, 327.
Ercoli, A. (1940). *Boll. sci. facolta chim. ind. Bologna*, p. 279.
—— (1941). *Hoppe-Seyl. Z.* **270**, 266.
Fieser, L. F. (1937). *The Chemistry of Natural Products related to Phenanthrene*, p. 251. New York: Reinhold Publishing Corporation.
Hughes, H. B. & Schmidt, L. H. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 162.
Mamoli, L. (1938). *Ber. dtsch. chem. Ges.* **71**, 2701.
Rosenheim, O. & Starling, W. W. (1933). *Chem. Ind.* **48**, 238.
Rosenheim, O. & Webster, T. A. (1943*a*). *Biochem. J.* **37**, 513.
—— —— (1943*b*). *Biochem. J.* **37**, 580.
Schmidt, L. H. & Hughes, H. B. (1942). *J. biol. Chem.* **143**, 771.
—— —— Green, M. H. & Cooper, E. (1942). *J. biol. Chem.* **145**, 228.
Sifferd, R. H. & Anderson, R. J. (1936). *Hoppe-Seyl. Z.* **239**, 270.
Turfitt, G. E. (1943). *Biochem. J.* **37**, 115.
—— (1944). *J. Bact.* **47**, 487.
Windaus, A. (1906). *Ber. dtsch. chem. Ges.* **39**, 2008.
—— Bursian, K. & Riemann, U. (1941). *Hoppe-Seyl. Z.* **271**, 177.

The Utilization of Urea in the Bovine Rumen

4. THE ISOLATION OF THE SYNTHESIZED MATERIAL AND THE CORRELATION BETWEEN PROTEIN SYNTHESIS AND MICROBIAL ACTIVITIES

By J. A. B. SMITH, *The Hannah Dairy Research Institute, Kirkhill, Ayr*
and F. BAKER (Plimmer Research Fellow), *The County Technical College, Guildford*

(*Received* 22 *September* 1944)

Pearson & Smith (1943) showed that when the liquid contents of the bovine rumen were incubated *in vitro* in the presence of certain carbohydrates a decrease in the non-protein nitrogen content of the liquid occurred. This decrease was assumed to result from protein synthesis, and since the rate of decrease could be controlled by the addition of toxic substances, the protein synthesis was attributed to

biological agencies. An investigation of the microbial population of the rumen was then undertaken, by direct microscopic examination of formolized samples. The micro-organisms observed were shown by Baker (1943) to be distributed amongst a number of representative classes. Using this information Baker demonstrated that, over the period during which the decrease in non-protein nitrogen occurred, microbiological normality was maintained, the flora remaining normal in types though increased in numbers. Pearson & Smith concluded that if the amount of protein apparently synthesized was even an approximate indication of the amount which can be synthesized under favourable conditions in the intact rumen, the non-protein nitrogen and carbohydrate of the diet are together capable of supplying an appreciable proportion of the ruminant's protein requirements. To establish this conclusion it was necessary to find (1) whether protein-containing material is actually formed in sufficient quantities to account for the observed decrease in non-protein nitrogen, (2) whether this material is formed by or incorporated in the micro-organisms of the rumen, and (3) whether the synthesized material can be assimilated during its passage through the gastro-intestinal tract.

The present paper embodies the results of experiments designed to meet these requirements by the isolation of the synthesized material in a form in which its microbial components can be identified and its biological value determined. In addition, an attempt has been made to elucidate some essential features of the microbial processes concerned, and to estimate the relative importance, as agents of synthesis, of the several classes of micro-organisms represented in the normal rumen.

METHODS

In vitro *incubations and analyses.* The methods of obtaining and incubating the samples of liquid rumen contents, and the general methods of analysis, were substantially the same as those described in a previous communication (Pearson & Smith, 1943).

Quantitative and qualitative microbiological examination. The fauna and flora of the rumen consist of: (1) Protozoa (oligotrich ciliates), (2) iodophile bacteria, i.e. bacteria which give a blue colour with iodine, and (3) aniodophile bacteria, i.e. bacteria which do not give a blue reaction with iodine.

The iodophile bacteria comprise: (*a*) large bacteria of the genera *Amylococcus*, *Amylosarcina*, *Amylobacterium*, *Amylospirillum* and *Oscillospira*, (*b*) small bacteria with no specifically distinct morphological characteristics (Baker, 1943). The Protozoa and the larger bacteria can be counted but not the other groups.

Counting procedure. The samples employed were made by diluting 25 ml. of rumen liquid with 75 ml. of approximately 3·5% formaldehyde solution. Each sample was well shaken and 1·0 ml. transferred to a flat-bottomed watch-glass. 1 ml. of Gram's iodine solution was added, the mixture vigorously agitated with a glass rod and a drop of standard size transferred without delay to a Burker cell with two counting chambers. Both chambers were filled simultaneously and two ruled areas were counted for each sample. The counts were made under an apochromatic 8 mm. lens with ×10 eyepiece, using a $\frac{3}{4}$ cone. Sixty $\frac{1}{25}$ mm. squares were counted in each chamber, making a total of 120 counts/sample. Micro-organisms projecting over the upper and left-hand borders were reckoned together with the contents of the square. The figures in the present paper represent the total number of a particular type of micro-organism (e.g. *Amylococcus*, *Amylospirillum*, etc.) in 120 squares, divided by 20; they are given to the nearest decimal place. The sum of these figures for the large iodophile bacteria represents the total macroiodophile count. In counting macroiodophiles only forms showing a strong iodine reaction were counted. Individual *Amylococcus* chains of more than three cells were reckoned as one unit. Chains of less than four cells were ignored. Each individual group of *Amylosarcina* or *Amylobacterium* and each *Oscillospira* trichome was reckoned as a unit. Every *Amylospirillum* of more than three turns was regarded as one unit. Forms of less than three turns were ignored. Made in this way the macroiodophile counts afford an index of the density of the members of the macroiodophile population which react with iodine to give a blue colour under the conditions specified. Other factors such as the synthesis and storage of starch by the bacteria may contribute to an increase in density in addition to cell multiplication. This is discussed in the interpretation of the results.

Volume of sediment. During the incubation of rumen contents the microflora tend to associate in zoogloea-like masses. The enumeration of individual micro-organisms in such masses is impracticable. The material can be readily sedimented, and the volume accumulating under different conditions affords an index of the amount of zoogloea formed and therefore of the combined bulk of the micro-iodophile and aniodophile bacteria. The figures recorded under this heading represent the volume of sediment obtained from 10 ml. of formolized sample when centrifuged for 1 min. at 1000 r.p.m.

Iodine reaction of the sediment. The increase in iodophile bacteria resulted in extensive synthesis of iodophillic polysaccharide. The intensity of the reaction of the sediment with iodine, therefore, afforded an independent macroscopic indication of the microbiological changes. The supernatant liquid was poured off from the sediment and the volume made up to 10 ml. with iodine solution. The mixture was well shaken and allowed to stand for a quarter of an hour. The iodine solution was then decanted and replaced by successive changes of water, centrifuging for 1 min. at 1000 r.p.m. after each change. The colour of the sediment was then examined against a white card.

EXPERIMENTAL AND RESULTS

The isolation of the protein-containing material

One of the first essentials was to find whether the protein-containing material (termed the 'sediment' throughout the remainder of this paper) could be readily isolated from the rumen liquor before and after incubation. For this purpose the following experiment was done.

A sample of rumen liquor was centrifuged for 15 min. at 2000 r.p.m., and the supernatant liquid divided into two

equal portions. (Centrifuging in this way was found in later experiments to give a supernatant liquid in which the amount of synthesis was readily determined.) One of the two portions was incubated with 1% maltose and 0·1% urea, the other with approximately 1% glycerol and 0·1% urea. Both incubations continued at 38° for 6 hr. Small samples were removed before and after incubation for the determination of non-protein nitrogen (N.P.N.) by the tungstate method, and also of pH and total nitrogen. 50 ml. were also removed and treated with 50 ml. of dilute formalin. The sediment in these formolized samples was isolated and examined on both a micro- and macro-scale. On the micro-scale 10 ml. samples were centrifuged at 3600 r.p.m. for 20 min. The supernatant layer was decanted into a Kjeldahl flask, the sediment mixed with 5 ml. dilute formalin, recentrifuged, and the second supernatant layer added to the first. Nitrogen was estimated in the supernatant liquid and also in the total sediment. On a large scale 50 ml. samples of the formolized samples were centrifuged at 3000 r.p.m. for 30 min. in weighed tubes. The supernatant layer was removed and the residue stirred with 20 ml. ethanol and recentrifuged. The residue from the second centrifugation was dried, weighed and its nitrogen content determined.

The more important results, recorded in Table 1, lead to the following conclusions. (1) As in the previous experiments (Pearson & Smith, 1943), maltose promoted synthesis whereas glycerol not only failed to do so but tended to cause protein hydrolysis. (2) The sediment containing the synthesized material can be isolated as an amorphous powder. (3) The amount of protein synthesis determined by isolating the sediment was of much the same order whether the isolation was on a micro- or macro-scale. In this particular experiment the synthesis found by isolating the sediment was slightly less than that estimated from the decrease in N.P.N. by the tungstate method.

Table 1. *The amount of synthesis or decomposition in rumen liquor estimated by isolating the dried sediment and by N.P.N. determinations*

(All values are recorded in mg./100 g. rumen liquor; increase is indicated by (+) and decrease by (−).)

	Large-scale treatment of formolized samples		Small-scale treatment of formolized samples		
Substance added	Change in weight of sediment during the incubation	Change in the protein-N of the sediment	N converted to protein, calculated from N.P.N. of supernatant layers	Change in the N of the sediment	Change in N.P.N. by sodium tungstate method
Maltose	+152·2	+6·7	+6·2	+6·4	−8·1
Glycerol	−16·5	−1·4	−0·2	−0·8	+0·7

The effect of different rates and times of centrifuging

Pearson & Smith (1943) have shown in one experiment that, when rumen liquor was centrifuged for 5 min. at 1000 r.p.m., the top layers of the supernatant liquid which contained great numbers of many different types of bacteria gave more synthesis than the bottom fractions which were not only rich in bacteria but also contained much vegetable matter and great numbers of Protozoa. This observation, together with the fact that the synthesized material could now be isolated in a crude form, led to experiments to find whether by centrifuging the rumen liquor for different times and at different speeds, a fraction could be obtained which would not only be relatively simple in its microbial population but would also possess relatively great synthesizing power. It was hoped that detailed biochemical and microbiological examination of such a fraction would give valuable information as to the nature of the agents which promote the synthesis and would yield a sediment rich in synthesized material whose biological value could then be determined. The following experiments were carried out.

A large sample of rumen liquor was strained through muslin and divided into four parts, *A*, *B*, *C* and *D*. *A* was incubated with 1% maltose and 0·1% urea for 5½ hr. at 38°. *B*, *C* and *D* were centrifuged for the times and speeds recorded in Table 2. The top fractions so obtained were then incubated under conditions identical with those for the incubation of *A*. Before and after incubation small samples were removed for the determination of pH, bacterial counts, total nitrogen and N.P.N. by the tungstate method. For the quantitative isolation of the 'sediment' 100 ml. samples were treated with 10 ml. formalin. These formolized samples were centrifuged for 30 min. at 3000 r.p.m. and the supernatant layers removed. The residues were stirred well with dilute formalin, the mixtures recentrifuged and the second batch of supernatant layers added to the first. The sediment was then washed twice with ethanol and once with ether, the mixtures being centrifuged after each washing. The ethanol-ether washings (*E*) were united and their nitrogen content determined. The sediment was dried overnight in a vacuum desiccator with P_2O_5 and finally in an oven at 100° for 1½ hr. It was then weighed. The aqueous supernatant liquors were heated for 10 min. at 100° with sufficient trichloroacetic acid to give a concentration of 10%. A precipitate (*F*) was formed which was filtered off and washed. This precipitate would contain any protein which was soluble in water or which was too finely dispersed to be centrifuged down with the sediment. The nitrogen contents of both the precipitate and the filtrate were determined. In this way the total nitrogen of the liquors before and after incubation was divided into 'sediment' nitrogen (*S*), nitrogen removed

Table 2. *Biochemical and microbiological results for incubations of rumen liquor before and after centrifuging*

(Nitrogen results are recorded in mg./100 g. rumen liquor.)

Centrifugation of rumen liquor ...	*A* Uncentrifuged rumen liquor			*B* 1000 r.p.m. for 3 min.			*C* 2000 r.p.m. for 15 min.			*D* 3000 r.p.m. for 25 min.		
	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change
pH	7·6	6·0	− 1·6	7·6	6·1	− 1·5	7·6	6·2	− 1·4	7·6	6·5	− 1·1
N removed by ethanol-ether (*E*)	3·2	5·3	+ 2·1	0·8	2·1	+ 1·3	0·4	1·7	+ 1·3	0·2	1·2	+ 1·0
Protein N not in 'sediment' but precipitated by trichloroacetic acid (*F*)	18·2	12·5	− 5·7	18·6	16·1	− 2·5	17·5	15·6	− 1·9	15·4	13·9	− 1·5
True N.P.N. not precipitated by trichloroacetic acid	55·3	40·5	−14·8	54·5	41·1	−13·4	57·9	43·1	−14·8	59·5	47·3	−12·2
Total N.P.N. as determined by tungstate method	60·1	43·5	−16·6	55·2	35·5	−19·7	56·7	38·1	−18·6	55·9	43·5	−12·4
Sediment nitrogen (*S*)	101·6	118·6	+17·0	11·3	29·0	+17·7	7·2	24·8	+17·6	3·2	16·0	+12·8
True synthesis of protein, i.e. change in *S* corrected for change in *F*	—	—	11·3	—	—	15·2	—	—	15·7	—	—	11·3
Weight of sediment, mg./100 g. liquor	1388	1615	+227	127	358	+231	77	366	+289	33	256	+222
% N in the dry sediment	7·3	7·3	—	8·9	8·1	− 0·8	9·3	6·8	− 2·5	9·7	6·3	− 3·4
Microbiological counts*												
Protozoa	17	12	− 5	0·6	0	− 0·6	0	0	0	0	0	0
Amylococcus chains	21	44	+23	9	27	+18	2	13	+11	0·6	5	+ 4·4
Amylospirillum	2	4	+ 2	2	3	+ 1	0	0	0	0	0	0
Amylobacterium	0·4	0·9	+ 0·5	0·3	0·8	+ 0·5	0	0	0	0	0	0
Total macroiodophile bacteria	23	49	+26	11	31	+20	2	13	+11	0·6	5	+ 4·4
Volume of zoogloea (ml.)	3·0	4·5	+ 1·5	0·1	0·5	+ 0·4	0·1	0·7	+ 0·6	0·1	0·5	+ 0·4
Iodine reaction	Some	Some		None	Strong		None	Strong		None	Strong	

* Total number per $60 \times \frac{1}{25}$ mm. squares/20 (see text).

from the wet sediment by ethanol-ether (*E*), protein nitrogen not contained in the sediment but precipitated by trichloroacetic acid (*F*), and true N.P.N. unprecipitated by trichloroacetic acid.

The results (Table 2) lead to the following conclusions:

(1) In all five incubations some of the protein nitrogen (*F*), not initially in the sediment but precipitated by trichloroacetic acid from the supernatant liquid, was either decomposed during incubation or was transferred to the sediment. This was particularly noticeable in *A* but much less so in *B*, *C* and *D*. To estimate the true amount of protein synthesis, the increase in sediment nitrogen (*S*) must be corrected for this change in *F*.

(2) The true protein synthesis attained a maximum of 15–16 mg. N/100 g. liquor in fractions *B* and *C*, where the liquor had been centrifuged for 3 min. at 1000 r.p.m. and for 15 min. at 2000 r.p.m. In the uncentrifuged material (*A*) and in the material centrifuged for 25 min. at 2000 r.p.m. (*B*) it was much less. It has been shown for uncentrifuged liquor (Pearson & Smith, 1943) that protein hydrolysis and synthesis appear to proceed concurrently and that either one or the other may predominate according to the conditions. It is probable, therefore, that as much synthesis actually occurred in *A* as in *B* and *C*, but that a greater amount of hydrolysis also occurred. That the synthesis in *D* was less than in *B* and *C* may have been due to the fact that the more intensive centrifuging had greatly reduced the number of micro-organisms initially present so that there were fewer to multiply.

(3) In the four incubations the actual weight by which the sediment increased was 0·22–0·29 g./100 g. rumen liquor. In *A*, the initial sediment was so great that this was equivalent to a change of only 16%, but in *B*, *C* and *D*, where the initial sediment was very much smaller, due to the fact that much of the suspended matter had been removed in the preliminary centrifuging, the weight of sediment was increased almost 3, 5 and 8 times respectively.

D gave a sediment of which at least 80% was synthesized during incubation.

(4) Synthesis in itself is obviously not dependent on the presence of Protozoa but is accompanied by a great increase in the numbers of micro-organisms, particularly iodophile types which synthesize polysaccharide and give an intense blue iodine reaction to the medium. In *D*, and to a less extent in *B* and *C*, the nitrogen percentage of the final sediment was less than in the initial. This suggests that the synthesis of protein is accompanied by an even greater synthesis of non-protein substances which, from other evidence, consist mainly of polysaccharide.

(5) As in the previous experiment the amount of synthesis calculated from the changes in N.P.N. by the tungstate method was generally higher than the synthesis shown to have occurred by isolating the 'sediment'.

Incubations without added carbohydrate and in the presence of glycerol, glucose or maltose

The technique just described for iodophile counts and also for arriving at the true synthesis by isolating the sediment and applying a correction for the difference in protein nitrogen precipitated by trichloroacetic acid was now applied to compare incubations in which no carbohydrate was added with those in which glycerol, glucose or maltose was present. Since in the previous experiment maximum synthesis was observed in a fraction which had been obtained by centrifuging at 2000 r.p.m. for 15 min., this speed and time were adopted in the present experiment. A large volume of centrifuged liquor was divided into four parts which were all incubated with 0·1% urea. One part contained no added carbohydrate; each of the other three contained 1% glycerol, 1% glucose or 1% maltose. Since the initial and final values for the chemical data were of a similar order to those recorded for incubations *B* and *C* in the previous experiment (Table 2), only the actual changes observed during incubation and microbiological data are recorded in Table 3. They lead to the following conclusions: (1) During incubation in the absence of added carbohydrate or with glycerol, hydrolysis tended to predominate. This was shown by a small increase in N.P.N. and by a small decrease in the weight of the sediment and in the nitrogen which it contained. With glucose and maltose, on the other hand, marked synthesis was observed. (2) Protein synthesis appeared to be accompanied by synthesis of polysaccharide. The fact that the nitrogen percentage in the final sediment was so much less than it was in the initial again suggests that the increase in polysaccharide may have been considerably greater than the increase in protein. (3) The iodophile counts, volume of zoogloea and iodine reaction greatly increased when synthesis predominated but decreased or showed little change when hydrolysis predominated. (4) The tungstate method of N.P.N. again gave results for protein synthesis which were higher than those found by isolating the sediment and correcting for protein nitrogen not contained in the sediment but precipitated by trichloroacetic acid (*F*).

In this experiment maltose and glucose gave rise to very similar amounts of protein synthesis, whereas in earlier work (Pearson & Smith, 1943) maltose was superior to glucose. The explanation for this difference may lie partly in the nature of the rumen sample used for incubation and partly in the fact that in the work now being discussed both sugars were present to the extent of 1% and incubation

Table 3. *Protein synthesis and iodophile counts for incubation in absence of added carbohydrate and in presence of glycerol, glucose or maltose*

(Nitrogen results are recorded in mg. N/100 g. rumen liquor.)

	No added carbohydrate		Glycerol		Glucose		Maltose	
Change in pH*	+ 0·1		− 0·1		− 1·2		− 1·2	
Change in protein N not in the sediment but precipitated by trichloroacetic acid (*F*)	− 1·9		+ 0·4		− 5·9		− 6·7	
Change in N.P.N. not precipitated by trichloroacetic acid together with the small amount of N removed by ethanol-ether from the sediment	+ 1·7		+ 1·3		−12·5		−11·7	
Change in N.P.N. (tungstate method)	+ 0·3		− 0·7		−15·1		−14·6	
Change in sediment N (*S*)	− 1·2		− 1·7		+18·6		+18·2	
True synthesis of protein (*S* corrected for *F*)	− 3·1		− 1·3		+12·7		+11·5	
Change in weight of sediment, mg./100 g. rumen liquor†	−15		−16		+346		+339	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
N in dry sediment (%)	10·3	10·6	10·7	10·8	10·5	6·9	10·3	6·8
Counts of total macroiodophile bacteria	2	0	3	0	2	53	2	50
Volume of zoogloea (ml.)	0·1	0·1	0·1	0·1	0·1	1·0	0·1	1·0
Iodine reaction	None	None	None	None	None	Strong	None	Strong

* The initial pH was 6·9.

† The initial sediments were respectively 146, 148, 149 and 146 mg./100 g. liquor.

proceeded for 2–3 hr., whereas in the earlier work their initial concentration was only 0·3 % and incubation continued for 5½ hr.

The correlation between synthesis and iodophile counts

The technique just described for making iodophile counts and estimating true synthesis by isolating the synthesized material and correcting for *F* was applied in three types of experiment to determine whether synthesis was always accompanied by an increase in the microbial population of the medium. One example of each type will be cited.

(*a*) *Changes in rumen liquor during incubation.* In the previous experiments samples were taken for examination and analysis before and after incubation for 5½ or 6 hr. In the present experiment

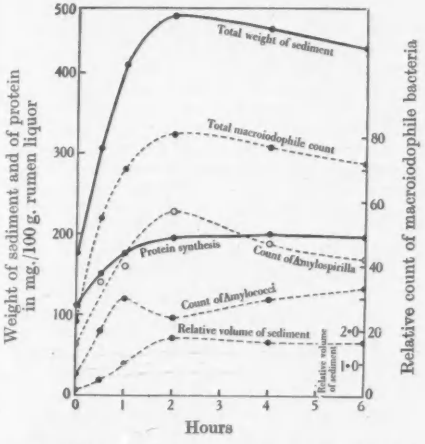


Fig. 1. Synthesis and macroiodophile counts estimated at intervals during an incubation of rumen liquor in the presence of added urea and maltose. The relative volume of sediment gives a measure of zoogloea formation.

samples were taken at the beginning and then after ½, 1, 2, 4 and 6 hr. so that a study could be made of the changes occurring as incubation proceeded. The results are illustrated in Fig. 1. The weight of sediment increased from 176 mg./100 g. rumen liquor initially to 495 mg. after 2 hr., while protein synthesis in the same period was equivalent to 16·3 mg. N or 102 mg. protein/100 g. rumen liquor. Thereafter the total weight of sediment decreased slightly while the amount of protein in the sediment remained almost constant, with a slight tendency to decrease towards the end of the 6 hr. The intensity of the iodine reaction (not shown in the figure) and the volume of sediment, which is a measure of the zoogloea formation, followed a course similar to that of the sediment weight. Up to 2 hr., a period in which the pH was within the limits normally encountered in fresh rumen contents, synthesis was accompanied by roughly corresponding increases in the iodophile population of the medium.

Since the total weight of sediment increased by 319 mg./100 g. rumen liquor in the first 2 hr., while the corresponding increase in protein was only 102 mg., it is evident that some substance was being elaborated much more rapidly than protein. Later, during the remaining 4 hr. when the sediment was tending to diminish, this substance tended to decrease slightly while the protein remained almost constant. The intensity of the iodine reaction at intervals during incubation suggested that the substance, which was synthesized and also hydrolyzed more rapidly than protein, was probably a starch-like polysaccharide which stained blue with iodine and which by direct microscopic examination could be seen to exist within the cells of the iodophile bacteria. This suggestion was confirmed by a further experiment, with an entirely different sample of rumen liquor in which the polysaccharide content of the sediment was estimated by the gravimetric cuprous oxide method after hydrolysis with HCl. The results recorded in Table 4 show that during

Table 4. *The increase of protein and polysaccharide during incubation for 6 hr.*

Duration of incubation (hr.)	Sediment (mg./100 g. rumen liquor)	N content of the sediment (%)	Protein content of the sediment ($N \times 6{\cdot}25$) (mg. protein/100 g. rumen liquor)	Polysaccharide content of the sediment (mg./100 g. rumen liquor)
0	49·4	8·1	25·0	6·4
1	69·3	6·7	28·8	18·4
2	98·0	6·3	38·8	28·0
4	162·4	6·5	65·6	62·0
6	196·2	6·1	75·0	78·6

the first hour the protein of the sediment increased by only 3·8 mg./100 ml. rumen liquor, whereas the polysaccharide increased by 12·0 mg. After 2 hr. the polysaccharide formation was almost twice as great as the protein formation, while the nitrogen content of the sediment, which was originally 8·1 %, had decreased to 6·3 %. By direct microscopical examination it can be observed that initially some of the iodophile bacteria are almost entirely depleted of the starch-like polysaccharide. It may be, therefore, that in the early stages of incubation in the presence of readily available sugar or starch the process of building up reserves of this polysaccharide greatly predominates over actual cell division. Later, when multiplication reaches a maximum and begins to decline, as it did after 2 hr. in the previous experiment (Fig. 1), the polysaccharide reserves tend to

diminish appreciably before any hydrolysis of protein can be observed.

(*b*) *The effect of different amounts of maltose.* A sample of rumen liquor which had been centrifuged for 10 min. at 2000 r.p.m. was divided into five portions. One portion was incubated without maltose and the others with amounts of maltose varying from 0·21 to 0·84 %. All five portions contained 0·1 % urea. Samples were analyzed at the beginning and after 4 hr. The results (Table 5) again show that protein synthesis is accompanied by a relatively large increase in the weight of sediment, in the volume of zoogloea and also in the iodophile count and in the intensity of the iodine reaction. Synthesis of both protein and polysaccharide increased with corresponding increases in the initial concentration of maltose. In the absence of maltose no protein synthesis occurred and the weight of sediment and iodophile count showed a small decrease.

Table 5. *Synthesis and iodophile counts for incubations in the presence of different concentrations of maltose*

	pH	True protein synthesis (mg. N/100 g. liquor)	Change in weight of sediment over the initial weight (mg./100 g. liquor)	N in the sediment (%)	Iodophile counts			Iodine reaction	Volume of zoogloea (ml.)
					Amylo-coccus	*Amylo-spirillum*	Total		
Mean initial values	7·0	—	—	10·1	1	1	2	Slight	0·1
Concentration of maltose (%)	Values after 4 hr. incubation								
None	7·6	0·3	−14	10·9	0	0	0	Slight	0·1
0·21	7·2	3·7	55	10·3	4	5	9	Strong	0·2
0·42	6·9	6·4	91	9·7	10	25	35	Strong	0·5
0·63	6·3	9·7	207	8·5	19	30	49	Very strong	0·5
0·84	5·8	11·5	358	7·4	24	35	59	Very strong	0·9

(*c*) *The effect of sodium fluoride.* In uncentrifuged rumen liquor increasing concentrations of NaF decrease the protein synthesis and bring about protein hydrolysis (Pearson & Smith, 1943). The following experiment was undertaken to determine whether the decreased synthesis caused in this way with centrifuged liquor was accompanied by a corresponding decrease in the weight of the sediment and in bacterial count. A sample of centrifuged liquor was divided into four portions, all of which were incubated for 3 hr. with 1 % maltose and 0·1 % urea. The first sample was incubated without NaF; the others contained 0·005, 0·1 and 0·5 % NaF respectively. The results are recorded in Table 6. With no NaF and with 0·005 % NaF protein synthesis was accompanied by an increase in sediment weight and iodophile count and by an intensification of the iodine reaction. When the protein hydrolysis predominated, as with 0·5 % NaF, the weight of sediment decreased slightly and there was no significant change in the iodophile count or in the intensity of the iodine reaction. With 0·1 % NaF intermediate values were obtained. These observations all indicate a general correlation between synthesis of protein and of polysaccharide on the one hand and multiplication of the iodophile population on the other.

Table 6. *Synthesis and iodophile counts in the presence of* NaF

	pH	Change in sediment N (mg./100 g. liquor)	Change in weight of sediment, compared with the initial value (mg./100 g. liquor)	Iodophile counts			Volume of zoogloea (ml.)	Iodine reaction
				Amylo-coccus	*Amylo-spirillum*	Total		
Mean initial values	6·1	—	—	2	1	3	0·5	Very weak
NaF (%)	Values after 3 hr. incubation							
None	5·5	13·7	311	17	3	20	1·0	Strong
0·005	5·6	12·7	303	15	4	19	1·0	Strong
0·1	5·7	2·8	106	8	2	10	0·5	Weaker
0·5	6·1	−5·7	−44	3	1	4	0·5	Very weak

In previous experiments (Pearson & Smith, 1943), 0·1% NaF caused very marked hydrolysis to predominate, whereas in the present experiment a small amount of synthesis was observed. This difference is almost certainly due to the fact that in the previous work uncentrifuged liquor was used. The large numbers of Protozoa and possibly also the quantities of vegetable matter present in uncentrifuged liquor result in a greatly increased protein hydrolysis in the presence of NaF.

Isolation of the sediment in amounts suitable for detailed examination

In order to prepare large samples of the sediment before and after incubation the following series of experiments were carried out.

Rumen liquor (2·5 l.) was centrifuged at 2000 r.p.m. for 10 min. and 1% maltose and 0·1% urea added to the supernatant liquid. One litre (*A*) of the liquid was then removed from the mixture for the preparation of the initial sediment and the remainder incubated at 38° for 4 hr. At the end of this period a second litre (*B*) was removed for the preparation of the final sediment. Formaldehyde (50 ml. of 36% (w/v)) was added to *A* and *B* to preserve them. In the present experiment some 10 l. of supernatant liquid were treated in this way. The formolized samples, 5 l. of *A* and 5 l. of *B*, were centrifuged for 30–40 min. at 3000 r.p.m. The sediments so obtained were transferred to volumetric flasks with water, made up to a known volume and the mixture well shaken. From both initial (*A*) and final (*B*) samples half the mixture was withdrawn by pipette and centrifuged. The sediment was washed twice with water and dried in a vacuum desiccator (method I). The other half was centrifuged, washed four times with ethanol, once with ether and then air-dried (method II). Method I gave a very dark green brittle glass-like substance which was readily reduced by pestle and mortar to a green-grey powder. In method II, the ethanol and ether removed the green colouring matter and left a white amorphous powder which was readily dried in air. The samples before incubation each amounted to approximately 1 g., containing 10·8% ash and just over 50% crude protein. After incubation the corresponding yield was 4 g., containing 5·3% ash and 33% crude protein. From the various results which have just been described, this relative decrease in the content of ash and protein can be ascribed mainly to an increase in polysaccharide. In another experiment, after incubation, a 7 g. sample of sediment was prepared by method II and analyzed. Polysaccharide was determined by hydrolyzing a portion of the material with dilute HCl and estimating the reducing sugar by the usual gravimetric cuprous oxide method. Lipoid matter was determined by saponification with ethanol-KOH, followed by ether extraction. Glucosazone (m.p. 203° decomp.) was readily prepared from the sediment after hydrolysis with HCl. No reducing sugar was present before hydrolysis.

The results of the analysis of the synthesized material are shown in Table 7, where they are compared with typical results recorded for linseed cake. The similarity in the general composition of the two products is most striking.

Table 7. *The analysis of the synthesized material from the rumen liquor compared with typical analytical figures for linseed cake*

	Rumen material (%)	Linseed cake (%)
Moisture	0·5	0·5
Ash	6·2	5·8
Crude protein (N × 6·25)	36·3	33·0
Total carbohydrate	46·6	50·0
Lipoids	9·5	10·7
	99·1	100·0

The values for the sediment are those found for a typical sample after washing it with ethanol and ether. The values for the linseed cake were obtained from values recorded in standard tables and corrected to 0·5% moisture.

Microscopical examination of dried sediment

Preparations of the dried sediment before and after incubation were examined microscopically to determine their microbiological characteristics, and to form a rough estimate of the kind and amount of plant residue which they contained.

Examination of the initial sediment prepared from unincubated material stained with iodine showed the presence of macroiodophile species in small numbers only, *Amylococcus* being the prevalent type. Most of the cocci were in pairs, chains being infrequent. Many of them did not react at all strongly with iodine. Individual fragments of the abundant zoogloea gave a pale yellow to brown reaction. These particular fragments appeared to be composed in large part of minute rods, cocci and vibrios which gave no blue colour with iodine. Similar micro-organisms were dispersed throughout the preparation.

Examination of the sediment from incubated material showed that pronounced changes had taken place. Macroiodophiles were present in very large numbers, the majority giving a dense, almost black, reaction. Some forms which did not give a blue colour with iodine were also observed. *Amylococcus* was again the prevalent type. Chains of 4–8 cells were frequently observed. *Amylospirillum*, *Amylosarcina*, and *Amylobacterium* were also identified. The iodine reaction of the zoogloea was much intensified. The micro-organisms present were similar to those in the unincubated sediment, but a great increase in the number of forms which reacted with iodine was apparent. Amongst such microiodophile species, coiled and interlacing chains of vibrios were conspicuous.

In order to detect fragments of vegetable material, the sediment was examined by polarizing microscope and in ultra-violet light. The latter method, which discloses the intrinsic fluorescence of encrusting substances, was found more convenient than the former, which makes use of the birefringence of cellulosic

structures. Preparations were made up in dilute ammonium hydroxide, which intensifies fluorescence. Examination of both incubated and unincubated material showed that plant residues were present only in very small amounts. Most of the fragments were identified as broken or detached epidermal hairs. In addition, isolated annular and small spiral vessels and pollen grains were observed. The proportion of plant residue to bacterial matter was greater in the unincubated than in the incubated material. In both sediments a few of the smaller kinds of ciliates could be detected.

These results entirely confirmed those obtained by the microscopic examination of formolized samples. They demonstrated also that the micro-organisms, of which the sediment is almost entirely composed, had undergone very little structural alteration in consequence of the procedures to which they had been subjected. Samples of these sediments are now being prepared from rumen liquor without addition of formalin so that their vitamin content and biological value may be determined.

DISCUSSION

Earlier experiments by Pearson & Smith (1943) showed that when rumen liquor is incubated under favourable conditions at 38°, the N.P.N. content of the mixture decreases while the total nitrogen remains constant. Pearson & Smith assumed therefore that protein was synthesized. The present experiments have confirmed this assumption and have shown that the decrease in N.P.N. is accompanied by the accumulation of a bulky sediment which gives an intense blue colour with iodine. This sediment is readily isolated and has been found to consist almost entirely of bacteria. It contains a starch-like polysaccharide and at least the main portion of the synthesized protein. In all experiments where protein synthesis occurred there was also a corresponding increase in the numbers of iodophile bacteria and a synthesis of polysaccharide. When the conditions were unfavourable to bacterial multiplication, i.e. in the absence of added carbohydrate or in the presence of NaF, neither protein nor polysaccharide synthesis was observed.

By centrifuging the rumen liquor before incubation, protein synthesis was shown to occur in the absence of Protozoa. The chief contributors to the protein synthesis are obviously the iodophile bacteria. They comprise a variety of macroiodophile and microiodophile species (Baker, 1943). Figures for the macroiodophiles indicate that *Amylococcus* was the predominating species in most of the incubations, though on occasion *Amylospirillum* and *Amylosarcina* reached comparable densities. Removal of the greater portion of the macroiodophiles by centrifuging showed, however, that the observed increases in the numbers of macroiodophiles in centrifuged liquor cannot account for more than a small fraction of the observed synthesis. From direct microscopical examination and other considerations there can be no doubt that the bulk of the synthesized polysaccharide and protein is incorporated in the small rods, cocci and vibrios of the microiodophile population.

Not all the organisms present in the sediment react with iodine. Among the non-reacting types are some, such as spirochaetes, which appear to synthesize starch-like polysaccharide. Direct observation shows that these do not accumulate in sufficient numbers to have any quantitative significance. Other non-reacting types, however, frequently attain a higher density. Except that they do not react with iodine, most of these are indistinguishable microscopically from the microiodophile population. Every transition can be observed between strongly reacting, weakly reacting and non-reacting forms. Moreover, observations on readily identifiable macroiodophile species have repeatedly shown that the amount of reacting polysaccharide present may vary within wide limits; forms replete with polysaccharide and forms devoid of it are encountered. By analogy, therefore, it is probable that most of the smaller non-reacting types do not belong to distinct aniodophile species, but that they represent dead or depleted members of the microiodophile population. Counts recorded in the present experiments for macroiodophiles show that the numbers of actually reacting types (i.e. those which stain blue with iodine), accumulating during incubation were greater than might reasonably be assumed to result from cell multiplication alone. It must be supposed therefore that many of the non-reacting forms present in the initial samples were still viable and that, in a proportion of these, re-storage of polysaccharide occurred in addition to cell division. This agrees with the chemical results, which showed that the synthesis of polysaccharide was greater than that of protein during the first 2 hr. of incubation, whereas towards the end of some incubations the polysaccharide-protein ratio and the iodine reaction both tended to decrease. The simplest conclusion is therefore that the bulk of the synthesized material is incorporated in the microiodophile population, but that the initial rate of synthesis and final rate of hydrolysis is greater for polysaccharide than for protein.

Since the greater part of the synthesis occurs within the first 2 hr. of incubation, while microbiological normality is maintained in the samples (Baker, 1943), it is reasonable to assume that in the intact rumen, the iodophile bacteria, which abound there, readily convert significant amounts of N.P.N. to protein, and of sugars (probably derived mainly from the starch in the diet) to polysaccharide, and

that by synthesizing protein they supply part of the ruminant's protein requirements. Experiments are still required to determine the biological value of the synthesized material and to assess the nutritive requirements of the bacterial agents of synthesis. Investigation along these lines is now in progress.

SUMMARY

1. Rumen liquor has been incubated *in vitro* with and without added carbohydrates. The synthesis which results under favourable conditions has been estimated by isolating the synthesized material as an amorphous powder which consists mainly of bacteria.

2. Synthesis of protein is accompanied by synthesis of a starch-like polysaccharide and by an increase in the iodophile population of the medium.

3. The greater portion of the synthesis is attributed to the masses of microiodophile bacteria which abound in the rumen. Protozoa do not appear to contribute to the synthesis.

4. Typical analytical figures for the sediment containing the synthesized material are 0·5 % moisture, 36·3 % protein, 46·6 % polysaccharide, 9·5 % lipoid matter and 6·2 % ash. These values are very similar to the corresponding values for a typical feeding stuff such as linseed cake.

The authors wish to thank Dr M. P. Applebey, Dr A. C. Thaysen and Dr N. C. Wright for their continued interest in the work. They also gratefully acknowledge the financial assistance kindly given by Imperial Chemical Industries Limited.

REFERENCES

Baker, F. (1943). *Ann. appl. Biol.* **30**, 230.

Pearson, R. M. & Smith, J. A. B. (1943). *Biochem. J.* **37**, 153.

INDEX to Vol. 38 December, 1944

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

F. DICKENS
E. J. KING
B. C. J. G. KNIGHT
J. R. P. O'BRIEN
A. R. TODD
F. G. YOUNG (*Chairman*)

CAMBRIDGE UNIVERSITY PRESS
ENGLAND

Price 2s. 6d. net

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. Under present conditions the parts of each volume will not necessarily appear at regular intervals but will be issued as frequently as possible. During 1945 it is hoped that five parts will be published.

The **Biochemical Society** was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Hon. Secs., Dr W. T. J. Morgan, Lister Institute, Chelsea Bridge Road, London, S.W.1, and Dr W. Robson, Dept. of Physiology, King's College, Strand, London, W.C.2, or from the Hon. Treas., Dr J. H. Bushill, The Laboratories, Cadby Hall, London, W.14.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £3. 10*s*. 0*d*. per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1, to Dr J. H. Bushill, The Laboratories, Cadby Hall, London, W.14, or to any bookseller. In the United States of America the *Journal* may be purchased from the University of Chicago Press, 5750 Ellis Avenue, Chicago, Ill., the American agents of the Cambridge University Press for the sale of the *Biochemical Journal*.

Communications respecting the sale of single issues or back numbers of the *Journal* should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

In paper covers

Volumes I–VI.	31*s*. 6*d*. net per vol.
Volumes VII–X.	(*Out of print*.)
Index of Authors and Subjects in Volumes I–X.	5*s*. 3*d*. net. (By post, 5*s*. 5*d*.)
Volumes XI–XVI.	(*Out of print*.)
Volumes XVII–XVIII.	60*s*. net per vol.
Volume XIX.	(*Out of print*.)
Volumes XX–XXV.	60*s*. net per vol.
Volumes XXVI–XXXVI.	70*s*. net per vol.
Index of Authors and Subjects in Volumes XI–XX.	10*s*. net. (By post, 10*s*. 4*d*.)

Binding. Quotations can be given by the publishers for bound copies of back numbers; also for buckram binding cases, and for binding subscribers' sets. (Suspended during the war.)

Index of Authors

Entries marked with an asterisk (*) are those for which only a title is given and of which there are no summaries. Small roman numerals refer to pages in Proceedings of the Biochemical Society.

ADAMS, G. A. *see* STANIER, R. Y. 168
*ANDERSON, A. B. Estimation of thio-uracil xv
ANDERSON, L. Obituary notice. Frank Lee Pyman, D.Sc., F.R.S. 283
ANDREWS, R. D. *see* CLAYTON, J. C. 452
AVINERI-SHAPIRO, S. *see* HESTRIN, S. 2

BACH, S. J. '*Q*' notation for expressing metabolic activities in tissue slices. Critical study 156
BACH, S. J., CROOK, E. M. and WILLIAMSON, S. On arginase and its participation in urea synthesis in liver 325
BACHARACH, A. L. and COATES, M. E. Vitamin P activity of some fruits and fruit products v
BACK, A. *see* FRANKENTHAL, L. 351
BACON, J. S. D., BALDWIN, E. and BELL, D. J. Glycogen. 9. Magnitude of 'unit chains' of liver glycogen of rabbits supplied with glucose, fructose and sucrose 198
BADER, R. and SCHÜTZ, F. Fractionation of fatty acids and bile acids from solutions of crude bile salts by adsorption on foam xxii
BADER, R. and SCHÜTZ, F. On temperature factor in adsorption on solid-liquid interface xx
*BADER, R., SCHÜTZ, F. and STACEY, M. Crystalline mucoprotein with high choline esterase activity xx
BAILEY, K. and WEBB, E. C. Purification and properties of yeast pyrophosphatase 394
BAKER, F. *see* SMITH, J. A. B. 496
BALDWIN, E. *see* BACON, J. S. D. 198
BARCROFT, J., MCANALLY, R. A. and PHILLIPSON, A. T. Absorption of acetic, propionic and butyric acids from alimentary canal iii
BARCROFT, J., MCANALLY, R. A. and PHILLIPSON, A. T. Destruction of acetic acid by beating heart iv
BARCROFT, J., MCANALLY, R. A. and PHILLIPSON, A. T. Production of acetic, propionic and butyric acids in alimentary canal ii
*BARTON-WRIGHT, E. C. Microbiological assay of nicotinic acid xix
BARTON-WRIGHT, E. C. Microbiological assay of nicotinic acid in cereals and other products 314
*BARTON-WRIGHT, E. C. Nicotinic acid content of cereals and other foods xix
BARTON-WRIGHT, E. C., EMERY, W. B. and ROBINSON, F. A. Isolation from liver of new factors essential for growth of *Lactobacillus helveticus* and *Streptococcus Lactis* R. xviii
BARTON-WRIGHT, E. C. *see also* MACRAE, T. F. 132
BELL, D. J. *see* BACON, J. S. D. 198
*BELL, G. H. and MCNAUGHT, M. L. Accuracy of haemoglobinimetry by Sahli method xv
BEVAN, H. G. L. *see* ROSE, F. L. 116
BEVERIDGE, J. M. R. and LUCAS, C. C. Analysis of hair keratin. 2. The dicarboxylic and basic amino-acids of human hair 88
BEVERIDGE, J. M. R. and LUCAS, C. C. Analysis of hair keratin. 3. Isolation of proline from human hair 95
BEVERIDGE, J. M. R. and LUCAS, C. C. Analysis of hair keratin. 4. Note on occurrence of serine in human hair 411
BIRKINSHAW, J. H., BRACKEN, A. and FINDLAY, W. P. K. Biochemistry of wood-rotting fungi. 4. Metabolic products of *Trametes suaveolens* (Linn.) Fr. 131
BLACKBURN, S., CONSDEN, R. and PHILLIPS, H. Action of sulphites on cystine disulphide linkages of wool. 4. Methylation of thiol groups of bisulphited wools 25
BLACKBURN, S. and PHILLIPS, H. Experiments on methylation and acetylation of wool, silk fibroin, collagen and gelatin 171
BLASCHKO, H. Reaction of anthocyanins with molybdate vi
BLASCHKO, H. and DUTHIE, R. Diamidines as inhibitors of enzyme action xxv
BOOTH, R. G. Cereal phosphatases. 1. Assay of free wheat phosphomonoesterase and characterization of free phosphatases of wheat 355
BOTTOMLEY, A. C. Chemical estimation of vitamin B_6 in foodstuffs v
BOWLER, R. G. Determination of thiocyanate in blood serum 385
BRACKEN, A. *see* BIRKINSHAW, J. H. 131
BRAY, H. G., GREGORY, J. E. and STACEY, M. Chemistry of tissues. 1. Chondroitin from cartilage 142
BRAY, H. G., HENRY, H. and STACEY, M. Observations on complex carbohydrates having blood-group specificity xxii
BRAY, H. G., SCHLÜCHTERER, E. and STACEY, M. Enzyme formation and polysaccharide synthesis by bacteria. 2. Polysaccharide formation by *Rhizobium radicicolum* strains 154
BRAY, H. G. and THORPE, W. V. Sampling of cooked cabbage for estimation of ascorbic acid in nutrition surveys xxiii
*BUNN, D. and MCFARLANE, A. S. Technique of preparative electrophoresis with large Tiselius U-tube xxxv
BUTLER, G. C. *see* MARRIAN, G. F. 322

CHANNON, H. J., MILLS, G. T. and WILLIAMS, R. T. Metabolism of 2:4:6-trinitrotoluene (α-T.N.T.) 70
CHATTAWAY, F. W., DOLBY, D. E. and HAPPOLD, F. C. Further observations on growth factors for *Lactobacillus casei* ϵ xviii
CHATTAWAY, F. W., HAPPOLD, F. C. and SANDFORD, M. A growth factor for *C. diphtheriae* present in liver 111
CLAYTON, J. C., HEMS, B. A., ROBINSON, F. A., ANDREWS, R. D. and HUNWICKE, R. F. Preparation of penicillin. Improved method of isolation 452
*CLEGHORN, R. A., FERGUSON, J. K. W. and HANNA, M. I. Serum protein and plasma volume changes in adrenal insufficiency vi
COATES, M. E. *see* BACHARACH, A. L. v
CONSDEN, R., GORDON, A. H. and MARTIN, A. J. P. Partition chromatography of amino-acids ix
CONSDEN, R., GORDON, A. H. and MARTIN, A. J. P. Qualitative analysis of proteins: partition chromatographic method using paper 224
CONSDEN, R. *see also* BLACKBURN, S. 25
COOK, R. P., DAVIDSON, W. A., KEAY, D. M. and MCINTOSH, D. G. Study of nutrition of group of boys aged 14–15 years xiii
COOK, R. P., KEAY, D. M. and MCINTOSH, D. G. Determination of plasma proteins and their value in group of boys xiv
COPPING, A. M. *see* MACRAE, T. F. 132
*COULSON, R. A., ELLINGER, P. and HOLDEN, M. Method for estimation of N-methyl-chloro-nicotinamide in urine v

COULSON, R. A., ELLINGER, P. and HOLDEN, M. Method for estimation of nicotinamide methochloride in urine 150
COULSON, R. A. *see also* ELLINGER, P. 265
CRAWFORD, T. B. B. and STOREY, I. D. E. Quantitative micro-method for separation of inorganic arsenite from arsenate in blood and urine 195
CROOK, E. M. and MORGAN, E. J. Reduction of dehydroascorbic acid in plant extracts 10
CROOK, E. M. *see also* BACH, S. J. 325
CURTIN, T. P. *see* REILLY, D. 37

DAVIDSON, J. N. and WAYMOUTH, C. Isolation and properties of ribonucleic acid from liver xiii
DAVIDSON, J. N. and WAYMOUTH, C. Nucleotide and polynucleotide content of liver tissue xiii
DAVIDSON, J. N. and WAYMOUTH, C. Tissue nucleic acids. 1. Ribonucleic acids and nucleotides in embryonic and adult tissue 39
DAVIDSON, J. N. and WAYMOUTH, C. Tissue nucleic acids. 2. Isolation and properties of liver ribonucleic acid 375
DAVIDSON, J. N. and WAYMOUTH, C. Tissue nucleic acids. 3. Nucleic acid and nucleotide content of liver tissue 379
DAVIDSON, W. A. *see* COOK, R. P. xiii
*DAVIS, J. G. and WARD, S. J. Apparatus for measuring abnormality of single quarter milk samples on farm xi
DAVIS, J. G. and WARD, S. J. Ultra-rapid examination of milk for chemical and bacteriological quality. (*a*) Single quarter samples at farm, (*b*) herd bulk samples on creamery platform ix
DAWSON, J. Effect of vitamin B_1 on histidine excretion in urine xvii
DELORY, G. E. and KING, E. J. 'Activation' of phosphatase 50
*DELORY, G. E. *see also* KING, E. J. vi
DELORY, G. E. *see also* KING, E. J. vii
DELORY, G. E. *see also* WATKINSON, J. iv
DOLBY, D. E. *see* CHATTAWAY, F. W. xviii
DUCKWORTH, J. *see* WARNOCK, G. M. 220
DUTHIE, R. *see* BLASCHKO, H. xxv

EGGLESTON, L. V. *see* KREBS, H. A. 426, xxix
ELLINGER, P. and COULSON, R. A. Urinary elimination of nicotinamide methochloride by man 265
ELLINGER, P. and HOLDEN, M. Quenching effect of electrolytes on fluorescence intensity of riboflavin and thiochrome 147
ELLINGER, P. *see also* COULSON, R. A. 150
*ELLINGER, P. *see also* COULSON, R. A. v
ELSDEN, S. R. and SYNGE, R. L. M. Starch as medium for partition chromatography ix
ELSON, L. A. and HOCH-LIGETI, C. Inhibition of enzyme systems by metabolic products of carcinogenic compounds. Urease and succinoxidase x
ELSON, L. A. and WARREN, F. L. Metabolism of azo compounds. 1. Azobenzene 217
ELSON, L. A. *see also* HADDOW, A. xxxiv, xxxv
EMERY, W. B. *see* BARTON-WRIGHT, E. C. xviii
EPPS, H. M. R. Studies on bacterial amino-acid decarboxylases. 2. *l*(−)-tyrosine decarboxylase from *Streptococcus faecalis* 242
EPPS, H. M. R. *see also* GALE, E. F. 232, 250

FAULKNER, G. H. Antibacterial action of some diphenyl derivatives 370
FEARON, W. R. Detection and estimation of uric acid by 2:6-dichloroquinone-chloroimide 399
*FERGUSON, J. K. W. *see* CLEGHORN, R. A. vi
FINDLAY, W. P. K. *see* BIRKINSHAW, J. H. 131
FRANKENTHAL, L. and BACK, A. Effect of X-rays on respiration of fowl erythrocytes 351
FRAZER, A. C. and SAMMONS, H. G. Formation of lower glycerides during hydrolysis of triglyceride with pancreatic lipase xx

GALE, E. F. and EPPS, H. M. R. Studies on bacterial amino-acid decarboxylases. 1. *l*(+)-lysine decarboxylase 232
GALE, E. F. and EPPS, H. M. R. Studies on bacterial amino-acid decarboxylases. 3. Distribution and preparation of codecarboxylase 250
GILCHRIST, E. *see* SCARBOROUGH, H. i
GILCHRIST, M. *see* KING, E. J. viii
GORDON, A. H., MARTIN, A. J. P. and SYNGE, R. L. M. Technical notes on partition chromatography of acetamino-acids with silica gel 65
GORDON, A. H. *see also* CONSDEN, R. 224, ix
GREGORY, J. E. *see* BRAY, H. G. 142
GUGGENHEIM, K. Biological value of carotene from various sources and effect of vitamin E on utilization of carotene and of vitamin A 260
GUGGENHEIM, K. and KOCH, W. Liver storage test for assessment of vitamin A 256

HADDOW, A., ELSON, L. A., RUDALL, K. M., TIMMIS, G. M. and ROE, E. M. F. Artificial induction of coat coloration in albino rat by means of 9-phenyl-5:6-benzo-*iso*-alloxazine xxxiv, xxxv
HADDOW, A. *see also* WATKINSON, J. iv
HALE, C. W. Studies on diffusing factors. 4. Action of reducing agents on hyaluronic acid and other polysaccharides 362
HALE, C. W. Studies on diffusing factors. 5. Influence of some environmental conditions on activity of hyaluronidase 368
HALL, T. C. and LEHMANN, H. Experiments on practicability of increasing calcium absorption with protein derivatives 117
*HANNA, M. I. *see* CLEGHORN, R. A. vi
HANSON, S. W. F., MILLS, G. T. and WILLIAMS, R. T. Study of determination of glucuronic acid by naphthoresorcinol reaction, with photoelectric absorptiometer 274
HAPPOLD, F. C. and WATERS, J. W. Action of clavatin on certain enzyme systems xvii
HAPPOLD, F. C. *see also* CHATTAWAY, F. W. 111, xviii
HARINGTON, C. R. and PITT RIVERS, R. V. Chemical conversion of diiodotyrosine into thyroxine xxxiv
HARINGTON, C. R. and PITT RIVERS, R. V. Mono-iodotyrosine 320
HARINGTON, C. R. and PITT RIVERS, R. V. Synthesis of cysteine-(cystine-) tyrosine peptides and action thereon of crystalline pepsin 417
HARRIS, L. J. and MAPSON, L. W. Determination of ascorbic acid in presence of interfering substances by 'continuous-flow' method. 1. Preliminary control tests xxiv
HARRIS, L. J. and MAPSON, L. W. Determination of ascorbic acid in presence of interfering substances by 'continuous-flow' method. 2. Tests with dehydrated vegetables, and with various raw and cooked fruits and vegetables xxv
HASLEWOOD, G. A. D. Metabolism of steroids. 4. Ketonic acids derived from cholic acid 108
HAWKINS, E. G. E. and HUNTER, R. F. 'Cyclization' of vitamin A and allied compounds 34
HEATLEY, N. G. Method for assay of penicillin 61
HEMS, B. A. *see* CLAYTON, J. C. 452
HENRY, H. Chemistry of Gram-staining xxi
HENRY, H. *see also* BRAY, H. G. xxii
HERBERT, F. K. Differentiation between prostatic phosphatase and other acid phosphatases in pathological human sera xxiii
HESTRIN, S. and AVINERI-SHAPIRO, S. Mechanism of polysaccharide production from sucrose 2
HILDITCH, T. P. and JASPERSON, H. Component acids of milk fats of goat, ewe and mare 443
HILDITCH, T. P. and MEARA, M. L. Human-milk fat. 1. Component fatty acids 29

HILDITCH, T. P. and MEARA, M. L. Human-milk fat. 2. Component glycerides 437
HINTON, J. J. C. Chemistry of wheat germ with particular reference to the scutellum 214
HOCH, H. Chromatographic investigation of β-carotene content of serum of the newborn infant 304
HOCH-LIGETI, C. *see* ELSON, L. A. x
HOLDEN, M. *see* COULSON, R. A. 150
*HOLDEN, M. *see also* COULSON, R. A. v
HOLDEN, M. *see also* ELLINGER, P. 147
HOLMAN, W. I. M. New method for oxidation of aneurin to thiochrome and a procedure for determination of aneurin in oats 388
HUGHES, D. E. *see* MCILWAIN, H. 187
HUMPHRIES, E. C. Studies on tannin compounds. 1. Changes during autolysis of minced cacao bean 182
HUNTER, R. F. and SCOTT, A. D. Palm oil carotenoids. 4. Proportion of α- to β-carotene in unripe and ripe palm oils and attempted interconversion of α- and β-carotenes 211
HUNTER, R. F., SCOTT, A. D. and WILLIAMS, N. E. Palm oil carotenoids. 3. Examination of the lipoid pigments present in 'Malay' and 'Bissao' palm oils 209
HUNTER, R. F. *see also* HAWKINS, E. G. E. 34
HUNWICKE, R. F. *see* CLAYTON, J. C. 452

JACOBY, F. C. and WOKES, F. Carotene and lycopene in rose hips and other fruits 279, i
JAMES, S. P. and SMITH, F. Chemistry of gum tragacanth xxi
JANZ, G. J. *see* de LOUREIRO, J. A. 16
JASPERSON, H. *see* HILDITCH, T. P. 443
JENKINS, G. N. Effect of mastication on ascorbic acid content of raw vegetables xix

KAPELLER-ADLER, R. Investigations on activity of the histaminase in normal and toxaemic pregnancy 270
KASSANIS, B. and KLECZKOWSKI, A. Effect of formaldehyde and mercuric chloride on tobacco mosaic virus 20
KAY, H. D. Obituary notice. Elfreida Constance Victoria Mattick (1887–1943) 1
KEAY, D. M. *see* COOK, R. P. xiii, xiv
KERLY, M. Riboflavin content of canteen meals 423
*KING, E. J. and DELORY, G. E. Formation of haematins and haemochromogens from crystalline haemin and haemoglobin vi
KING, E. J. and DELORY, G. E. Simple step photometer for field and emergency use vii
KING, E. J., GILCHRIST, M. and WHITE, J. C. Case of methaemoglobinaemia viii
KING, E. J. and NAGELSCHMIDT, G. Mineral content of lungs of workers from South Wales coalfield vi
KING, E. J. *see also* DELORY, G. E. 50
KING, E. J. *see also* WATKINSON, J. iv
*KING, E. J. *see also* YOUNG, J. vi
KING, H. K. and MORGAN, W. T. J. Isolation of blood group A substance from pseudo-mucinous ovarian cyst fluids x
KLECZKOWSKI, A. Combination of potato virus *X* and tobacco mosaic virus with pepsin and trypsin 160
KLECZKOWSKI, A. *see also* KASSANIS, B. 20
KLEINZELLER, A. Fat formation in *Torulopsis lipofera* 480
KNOX, R. and POLLOCK, M. R. Bacterial tetrathionase: adaptation without demonstrable cell growth 299
KOCH, W. *see* GUGGENHEIM, K. 256
KOSTERLITZ, H. W. Effect of dietary protein on liver cytoplasm xiv
KREBS, H. A. Enzymic hydrolysis of patulin xxix
KREBS, H. A. and EGGLESTON, L. V. Metabolism of acetoacetic acid xxix
KREBS, H. A. and EGGLESTON, L. V. Micro-determination of *iso*citric and *cis*-aconitic acid in biological material 426

LASER, H. Oxidative metabolism of *Ascaris suis* 333
*LASNITZKI, A. and WOODHOUSE, D. L. Haemolymphatic changes in lymph-nodes of rat, obtained (1) after prolonged administration and (2) shortly after single injection of 1:2:5:6-dibenzanthracene xxii
LEHMANN, H. *see* HALL, T. C. 117
LEIBOWITZ, J. Isolation of trehalose from desert manna 205
LIDDELL, H. F. and RYDON, H. N. New indicator for use in partition chromatography: 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine 68
LONG, C. Stabilization and estimation of pyruvic acid in blood samples 447, xxvii
LONG, T. *see* REILLY, D. 37
de LOUREIRO, J. A. and JANZ, G. F. Iodometric and colorimetric methods for estimation of calcium in serum based on use of improved permanganate solution 16
LUCAS, C. C. *see* BEVERIDGE, J. M. R. 88, 95, 411
LUGG, J. W. H. and WELLER, R. A. Large-scale extraction of protein samples reasonably representative of the whole proteins in leaves of some plants: amide, tyrosine, tryptophan, cystine (plus cysteine) and methionine contents of the preparations 408

MCANALLY, R. A. *see* BARCROFT, J. ii, iii, iv
MCCANCE, R. A. Chemistry of growth and food value of common eel (*Anguilla anguilla* (L.)) 474
MCFARLANE, A. S. and RIMINGTON, C. Electrophoresis of taka-diastase preparations xxxiii
*MCFARLANE, A. S. *see also* BUNN, D. xxxv
MCILWAIN, H. Absence of gross displacement of pantothenate and *p*-aminobenzoate from micro-organisms, by pantoyltaurine and sulphanilamide viii
MCILWAIN, H. Biochemical characterization of the actions of chemotherapeutic agents. 1. Measurement of growth of streptococcal cultures through their gaseous metabolism, and effects of pantothenate and pantoyltaurine upon metabolism and growth 97
MCILWAIN, H. Metabolic definition of drug-receptor xxviii
MCILWAIN, H. and HUGHES, D. E. Biochemical characterization of actions of chemotherapeutic agents. 2. A reaction of haemolytic streptococci, involving pantothenate-usage, inhibited by pantoyltaurine, and associated with carbohydrate metabolism 187
MCINTOSH, D. G. *see* COOK, R. P. xiii, xiv
*MCNAUGHT, M. L. *see* BELL, G. H. xv
MACRAE, T. F., BARTON-WRIGHT, E. C. and COPPING, A. M. Riboflavin content of food served in Royal Air Force messes 132
MADINAVEITIA, J. Antagonism of some antimalarial drugs by riboflavine xxvii
MADINAVEITIA, J., MARTIN, A. R., ROSE, F. L. and SWAIN, G. Antibacterial activity of some antagonists of pantothenic acid xxvii
MADINAVEITIA, J. and STACEY, M. Substrates for hyaluronidase 413
MANN, T. Studies on metabolism of mould fungi. 1. Phosphorus metabolism in moulds 339
MANN, T. Studies on metabolism of mould fungi. 2. Isolation of pyrophosphate and metaphosphate from *Aspergillus niger* 345
MAPSON, L. W. *see* HARRIS, L. J. xxiv, xxv
MARRIAN, G. F. and BUTLER, G. C. Isolation of a Δ^5-androstene-3 (β), 16, 17-triol from urine of normal human males and females 322
MARTIN, A. J. P. *see* CONSDEN, R. 224, ix
MARTIN, A. J. P. *see also* GORDON, A. H. 65
MARTIN, A. R. and ROSE, F. L. Antibacterial activity of substances related to 4-aminobenzoic acid xxviii
MARTIN, A. R. *see also* MADINAVEITIA, J. xxvii
MEARA, M. L. *see* HILDITCH, T. P. 29, 437
MILLS, G. T. *see* CHANNON, H. J. 70
MILLS, G. T. *see also* HANSON, S. W. F. 274
MOHAMMED, AHMED HASSAN. Notes on toxins of Egyptian scorpions 284

MOORE, T. *see* TOŠIĆ, J. xxvi
MORGAN, E. J. *see* CROOK, E. M. 10
MORGAN, W. T. J. *see* KING, H. K. x
MORRIS, C. J. O. R. Chromatographic determination of Evans blue in plasma and serum 203

NAGELSCHMIDT, G. *see* KING, E. J. vi
NEUBERGER, A. Reaction between histidine and formaldehyde 309
NEUBERGER, A. and SANGER, F. Availability of ε-acetyl-*d*-lysine and ε-methyl-*dl*-lysine for growth 125
NEUBERGER, A. and SANGER, F. Metabolism of lysine 119
*NEUBERGER, A. and WEBSTER, T. A. Lysine requirement of adult rat xxxv
NIERENSTEIN, M. and SWANTON, A. Colouring matters of galls 373

OXFORD, A. E. Diplococcin, anti-bacterial protein elaborated by certain milk streptococci 178

PETTIGREW, F. W. *see* STEDMAN, E. xxxi
PHILLIPS, H. *see* BLACKBURN, S. 25, 171
PHILLIPSON, A. T. *see* BARCROFT, J. ii, iii, iv
PITT RIVERS, R. V. *see* HARINGTON, C. R. 320, 417, xxxiv
PLIMMER, R. H. A. Van Slyke's copper sulphate method for measuring specific gravities of whole blood, plasma and serum vii
POLLOCK, M. R. *see* KNOX, R. 299
PREECE, I. A. Some hemicellulose extraction methods 402

RAMSAY, W. N. M. Ferrihaemoglobinaemia (methaemoglobinaemia) in man and horse 470
RAMSAY, W. N. M. Micro-volumetric determination of iron, with particular reference to blood 467
REILLY, D., LONG, T. and CURTIN, T. P. Influence of sodium nitrate concentration on metabolism of *Penicillium sclerotiorum* van Beyma 37
RIMINGTON, C. *N*-acetylglucosaminidase in commercial taka-diastase preparations xxxii
RIMINGTON, C. and ROWLANDS, I. W. Serum gonadotrophin. 2. Further purification of the active material 54
RIMINGTON, C. *see also* MCFARLANE, A. S. xxxiii
ROBINSON, F. A. *see* BARTON-WRIGHT, E. C. xviii
ROBINSON, F. A. *see also* CLAYTON, J. C. 452
ROE, E. M. F. *see* HADDOW, A. xxxiv, xxxv
ROGERS, H. J. Conditions controlling production of hyaluronidase by different types of micro-organisms xxxiii
ROSE, F. L. and BEVAN, H. G. L. New coupling component and simplified method for estimation of sulphanilamide drugs 116
ROSE, F. L. *see also* MADINAVEITIA, J. xxvii
ROSE, F. L. *see also* MARTIN, A. R. xxviii
ROWLANDS, I. W. *see* RIMINGTON, C. 54
RUDALL, K. M. *see* HADDOW, A. xxxiv, xxxv
RYDON, H. N. *see* LIDDELL, H. F. 68

SAMMONS, H. G. *see* FRAZER, A. C. xx
SANDFORD, M. *see* CHATTAWAY, F. W. 111
SANGER, F. *see* NEUBERGER, A. 119, 125
SCARBOROUGH, H. and GILCHRIST, E. Observations on correlation between capillary resistance and ascorbic acid in plasma i
SCHLÜCHTERER, E. *see* BRAY, H. G. 154
*SCHÜTZ, F. *see* BADER, R. xx
SCHÜTZ, F. *see also* BADER, R. xx, xxii
SCOTT, A. D. *see* HUNTER, R. F. 209, 211
SHRIKHANDE, J. G. Use of bromine in estimation of cellulose in plant materials 207
SMITH, F. *see* JAMES, S. P. xxi
SMITH, J. A. B. and BAKER, F. Utilization of urea in bovine rumen. 4. Isolation of the synthesized material and correlation between protein synthesis and microbial activities 496
SNOW, G. A. and ZILVA, S. S. Critical examination of Lugg's method for determination of *l*-ascorbic acid. 2 458
*STACEY, M. *see* BADER, R. xx
STACEY, M. *see also* BRAY, H. G. 142, 154, xxii
STACEY, M. *see also* MADINAVEITIA, J. 413
STANIER, R. Y. and ADAMS, G. A. Nature of *Aeromonas* fermentation 168
STEDMAN, E. and STEDMAN, ELLEN. Nature of basic proteins of salmon cell-nuclei and its bearing on chemical composition of chromosomes xxvi
STEDMAN, E., STEDMAN, ELLEN and PETTIGREW, F. W. Retardation of growth of mouse carcinoma 2146 by histone and protamine xxxi
STEDMAN, ELLEN, *see* STEDMAN, E. xxvi, xxxi
STOREY, I. D. E. *see* CRAWFORD, T. B. B. 195
STRELITZ, F. Studies on cholinesterase. 4. Purification of pseudo-cholinesterase from horse serum 86
SWAIN, G. *see* MADINAVEITIA, J. xxvii
SWANTON, A. *see* NIERENSTEIN, M. 373
SYKES, W. O. Observations on metabolism of sulphamezathine in intact body and in isolated tissues xxix
SYNGE, R. L. M. Analysis of partial hydrolysate of gramicidin by partition chromatography with starch 285
SYNGE, R. L. M. Isolation of ethanolamine from acid hydrolysates of gramicidin xxxi
SYNGE, R. L. M. *see also* ELSDEN, S. R. ix
SYNGE, R. L. M. *see also* GORDON, A. H. 65

THOMPSON, R. H. S. and WHITTAKER, V. P. Esterases of skin 295
THORPE, W. V. *see* BRAY, H. G. xxiii
TIMMIS, G. M. *see* HADDOW, A. xxxiv, xxxv
TOŠIĆ, J. and MOORE, T. Chemical estimation of vitamin E xxvi
TURFITT, G. E. Microbiological degradation of steroids. 2. Oxidation of cholesterol by *Proactinomyces* spp. 492

WARD, S. J. *see* DAVIS, J. G. ix
*WARD, S. J. *see also* DAVIS, J. G. xi
WARNOCK, G. M. and DUCKWORTH, J. Changes in skeleton during gestation and lactation in rat 220
WARREN, F. L. *see* ELSON, L. A. 217
WATERS, J. W. *see* HAPPOLD, F. C. xvii
WATKINSON, J., DELORY, G. E., KING, E. J. and HADDOW, A. Plasma acid phosphatase in carcinoma of prostate and effect of treatment with stilboestrol iv
WAYMOUTH, C. *see* DAVIDSON, J. N. 39, 375, 379, xiii
WEBB, E. C. *see* BAILEY, K. 394
*WEBSTER, T. A. *see* NEUBERGER, A. xxxv
WEIL-MALHERBE, H. Fluorimetric estimation of 3:4-benzpyrene in whole mice 135
WEIL-MALHERBE, H. Note on fluorimetric estimation of 3:4-benzpyrene by method of Miller and Baumann 141
WELLER, R. A. *see* LUGG, J. W. H. 408
WEST, C. and ZILVA, S. S. Synthesis of vitamin C in stored apples 105
WHITE, J. C. *see* KING, E. J. viii
*WHITE, P. B. Assay of penicillin xxxv
WHITTAKER, V. P. *see* THOMPSON, R. H. S. 295
WILLIAMS, N. E. *see* HUNTER, R. F. 209
WILLIAMS, R. T. *see* CHANNON, H. J. 70
WILLIAMS, R. T. *see also* HANSON, S. W. F. 274
WILLIAMSON, S. *see* BACH, S. J. 325
WOKES, F. *see* JACOBY, F. C. 279, i
*WOOD, E. J. *see* YOUNG, J. vi
WOODHOUSE, D. L. *see* LASNITZKI, A. xxii

*YOUNG, J., WOOD, E. J. and KING, E. J. Nutritional survey among pregnant women vi

ZILVA, S. S. *see* SNOW, G. A. 458
ZILVA, S. S. *see also* WEST, C. 105

Index of Subjects

Entries marked with an asterisk (*) are those for which only a title is given and of which there are no summaries

Absorptiometer, photoelectric, determination of glucuronic acid with (Hanson, Mills & Williams) 274
Absorption, of acetic, propionic and butyric acids (Barcroft, McAnally & Phillipson) iii
Absorption, of calcium, effect of amino-acids on (Hall & Lehmann) 117
Acetamino-acids, chromatography of (Gordon, Martin & Synge) 65
Acetic acid, absorption of (Barcroft, McAnally & Phillipson) iii
Acetic acid, destruction of, by heart (Barcroft, McAnally & Phillipson) iv
Acetic acid, production of, in alimentary canal (Barcroft, McAnally & Phillipson) ii
Acetoacetic acid, metabolism of (Krebs & Eggleston) xxix
Acetylation, of wool, silk fibroin, collagen and gelatin (Blackburn & Phillips) 171
N-Acetylglucosaminidase, in taka-diastase (Rimington) xxxii
ε-Acetyl-*d*-lysine, and growth (Neuberger & Sanger) 125
Acid phosphatases, in sera, differentiation of (Herbert) xxiii
cis-Aconitic acid, in biological material, micro-determination of (Krebs & Eggleston) 426
*Adrenal insufficiency, serum protein and plasma volume changes in (Cleghorn, Ferguson & Hanna) vi
Adsorption on foam, fractionation of fatty and bile acids from solutions of crude bile salts by (Bader & Schütz) xxii
Adsorption, on solid-liquid interface, temperature factor in (Bader & Schütz) xx
Aeromonas hydrophila, fermentation by (Stanier & Adams) 168
Alimentary canal, absorption of acetic, propionic and butyric acids from (Barcroft, McAnally & Phillipson) iii
Alimentary canal, production of acetic, propionic and butyric acids in (Barcroft, McAnally & Phillipson) ii
Amide content, of plant leaf proteins (Lugg & Weller) 408
Amino-acid decarboxylases, from bacteria (Gale & Epps) 232, 250
Amino-acid decarboxylases, from bacteria (Epps) 242
Amino-acid, lysine, metabolism of (Neuberger & Sanger) 119
Amino-acids, basic, of human hair (Beveridge & Lucas) 88
Amino-acids, chromatography of (Consden, Gordon & Martin) ix
Amino-acids, effect of, on absorption of calcium (Hall & Lehmann) 117
Amino-acids, histidine, reaction of, with formaldehyde (Neuberger) 309
Amino-acids, of plant leaf proteins (Lugg & Weller) 408
p-Aminobenzoate, non-displacement of, from micro-organisms, by sulphanilamide (McIlwain) viii
4-Aminobenzoic acid, derivatives of, antibacterial activity of (Martin & Rose) xxviii
Androstenetriol, in normal urine (Marrian & Butler) 322
Δ^5-Androstene-3 (β), 16, 17-triol, in normal urine (Marrian & Butler) 322
Aneurin, *see also* Vitamin B_1
Aneurin, in oats, determination of (Holman) 388
Aneurin, oxidation of, to thiochrome (Holman) 388
Anguilla anguilla (L.), chemistry of growth and food value of (McCance) 474
Anthocyanins, reaction of, with molybdate (Blaschko) vi
Antibacterial action of diphenyl derivatives (Faulkner) 370
Antibacterial activity, of derivatives of 4-aminobenzoic acid (Martin & Rose) xxviii
Anti-bacterial protein, produced by milk streptococci (Oxford) 178
Antimalarial drugs, antagonism between riboflavin and (Madinaveitia) xxvii
Apples, synthesis of vitamin C in (West & Zilva) 105
Arginase activity, of liver slices (Bach) 156
Arginase, and urea synthesis (Bach, Crook & Williamson) 325
Arsenate, in blood and urine, separation of, from arsenite (Crawford & Storey) 195
Arsenite, in blood and urine, separation of, from arsenate (Crawford & Storey) 195
Ascaris suis, oxidative metabolism of (Laser) 333
Ascorbic acid, *see also* Vitamin C
Ascorbic acid content, of raw vegetables, effect of mastication on (Jenkins) xix
Ascorbic acid, dehydro-, reduction of (Crook & Morgan) 10
Ascorbic acid, determination of, in presence of interfering substances (Harris & Mapson) xxiv, xxv
Ascorbic acid, estimation of, in cooked cabbage (Bray & Thorpe) xxiii
l-Ascorbic acid, Lugg's method for determination of (Snow & Zilva) 458
Ascorbic acid, in plasma, and capillary resistance, correlation between (Scarborough & Gilchrist) i
Aspergillus niger, pyrophosphate and metaphosphate from (Mann) 345
Autolysis, of cacao bean tannins (Humphries) 182
Azobenzene, metabolism of (Elson & Warren) 217
Azo compounds, metabolism of (Elson & Warren) 217

Bacteria, amino-acid decarboxylases from (Gale & Epps) 232, 250
Bacteria, amino-acid decarboxylases from (Epps) 242
Bacteria, codecarboxylase in (Gale & Epps) 250
Bacteria, enzyme and polysaccharide formation (Bray, Schlüchterer & Stacey) 154
Bacteria, *l*(+)-lysine decarboxylase from (Gale & Epps) 232
Bacteria, *l*(−)-tyrosine decarboxylase from (Epps) 242
Bacterial tetrathionase, adaptation without cell growth (Knox & Pollock) 299
Bacteriostatic protein, produced by milk streptococci (Oxford) 178
Basic proteins, of salmon cell-nuclei (Stedman & Stedman) xxvi
3:4-Benzpyrene, estimation of (Weil-Malherbe) 135, 141
Bile acids, fractionation of, from solutions of crude bile salts, by adsorption on foam (Bader & Schütz) xxii
Bile salts, solutions of, fractionation of fatty and bile acids from (Bader & Schütz) xxii
Bisulphited wool, methylation of thiol groups of (Blackburn, Consden & Phillips) 25
Blood, determination of specific gravity of (Plimmer) vii
Blood group A substance, isolation of, from pseudo-mucinous ovarian cyst fluids (King & Morgan) x
Blood-group specificity, of complex carbohydrates (Bray, Henry & Stacey) xxii

Blood, micro-determination of iron in (Ramsay) 467
Blood, pyruvic acid in, stabilization and estimation of (Long) 447, xxvii
Blood, separation of arsenite from arsenate in (Crawford & Storey) 195
Blood serum, thiocyanate in, determination of (Bowler) 385
Bromine, use of, in determination of cellulose (Shrikhande) 207
Butyric acid, absorption of (Barcroft, McAnally & Phillipson) iii
Butyric acid, production of, in alimentary canal (Barcroft, McAnally & Phillipson) ii

Cabbage, cooked, estimation of ascorbic acid in (Bray & Thorpe) xxiii
Cacao bean, changes in tannins of, during autolysis (Humphries) 182
Calcium absorption, effect of amino-acids on (Hall & Lehmann) 117
Calcium, in serum, estimation of (de Loureiro & Janz) 16
Capillary resistance, and plasma ascorbic acid, correlation between (Scarborough & Gilchrist) i
Carbohydrates, complex, blood-group specificity of (Bray, Henry & Stacey) xxii
Carbohydrates, fermentation of, by *Aeromonas hydrophila* (Stanier & Adams) 168
Carcinogens, metabolic products of, inhibition of urease and succinoxidase by (Elson & Hoch-Ligeti) x
Carcinoma, mouse, retardation of growth of, by histone and protamine (Stedman, Stedman & Pettigrew) xxxi
Carcinoma, of prostate, effect of stilboestrol on plasma acid phosphatase in (Watkinson, Delory, King & Haddow) iv
Carotene, biological value of (Guggenheim) 260
α-Carotene, in palm oils (Hunter, Scott & Williams) 209
α-Carotene, in palm oils (Hunter & Scott) 211
β-Carotene, in palm oils (Hunter, Scott & Williams) 209
β-Carotene, in palm oils (Hunter & Scott) 211
γ-Carotene, in palm oils (Hunter, Scott & Williams) 209
γ-Carotene, in palm oils (Hunter & Scott) 211
Carotene, in rose hips and other fruits (Jacoby & Wokes) 279, i
β-Carotene, in serum of infants (Hoch) 304
Carotene, utilization of, effect of vitamin E on (Guggenheim) 260
Carotenoids, in palm oils (Hunter, Scott & Williams) 209
Carotenoids, in palm oils (Hunter & Scott) 211
Cartilage, chondroitin from (Bray, Gregory & Stacey) 142
Cell-nuclei, of salmon, basic proteins of (Stedman & Stedman) xxvi
Cellulose, determination of, in plants (Shrikhande) 207
Cereals, microbiological assay of nicotinic acid in (Barton-Wright) 314
*Cereals, nicotinic acid content of (Barton-Wright) xix
Cereals, phosphatases in (Booth) 355
Chain-length, of different glycogens (Bacon, Baldwin & Bell) 198
Chemotherapeutic agents, actions of (McIlwain & Hughes) 187
Chemotherapeutic agents, biochemical characterization of actions of (McIlwain) 97
Cholesterol, oxidation of, by *Proactinomyces* spp. (Turfitt) 492
Cholic acid, derivation of ketonic acids from (Haslewood) 108
*Choline esterase activity, of crystalline mucoprotein (Bader, Schütz & Stacey) xx
Cholinesterase, pseudo-, from horse serum, purification of (Strelitz) 86
Chondroitin, from cartilage (Bray, Gregory & Stacey) 142
Chromatography, of amino-acids (Consden, Gordon & Martin) ix
Chromatography, of β-carotene, in serum of infants (Hoch) 304
Chromatography, 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine as indicator in (Liddell & Rydon) 68
Chromatography, of Evans blue in plasma and serum (Morris) 203
Chromatography, of proteins, with paper (Consden, Gordon & Martin) 224
Chromatography, with silica gel, of acetamino-acids (Gordon, Martin & Synge) 65
Chromatography, with starch, of gramicidin hydrolysate (Synge) 285
Chromatography, starch as medium for (Elsden & Synge) ix
Chromosomes, chemical composition of (Stedman & Stedman) xxvi
*iso*Citric acid, in biological material, micro-determination of (Krebs & Eggleston) 426
Clavatin, action of, on enzyme systems (Happold & Waters) xvii
Codecarboxylase, preparation of (Gale & Epps) 250
Codecarboxylase, in tissues, yeasts and bacteria (Gale & Epps) 250
Collagen, methylation and acetylation of (Blackburn & Phillips) 171
Colouring matters, of galls (Nierenstein & Swanton) 373
Copper sulphate method, for measuring specific gravities of blood, plasma and serum (Plimmer) vii
Corynebacterium diphtheriae, growth factor for, from liver (Chattaway, Happold & Sandford) 111
'Cyclization', of vitamin A (Hawkins & Hunter) 34
Cysteine-(cystine-) tyrosine peptides, action of crystalline pepsin on (Harington & Pitt Rivers) 417
Cysteine-(cystine-) tyrosine peptides, synthesis of (Harington & Pitt Rivers) 417
Cystine content, of plant leaf proteins (Lugg & Weller) 408
Cystine disulphide linkages, of wool, action of sulphites on (Blackburn, Consden & Phillips) 25
Cytoplasm of liver, effect of dietary protein on (Kosterlitz) xiv

Decarboxylases, of amino-acids, from bacteria (Gale & Epps) 232, 250
Decarboxylases, of amino-acids, from bacteria (Epps) 242
Dehydroascorbic acid, in plant extracts, reduction of (Crook & Morgan) 10
Desert manna, isolation of trehalose from (Leibowitz) 205
Diamidines, inhibition of enzymes by (Blaschko & Duthie) xxv
*1:2:5:6-Dibenzanthracene, haemolymphatic changes in lymph-nodes of rat, after administration of (Lasnitzki & Woodhouse) xxii
Dicarboxylic acids, of human hair (Beveridge & Lucas) 88
2:6-Dichloroquinone-chloroimide, uric acid estimation by (Fearon) 399
Dietary protein, effect of, on liver cytoplasm (Kosterlitz) xiv
Diffusing factors (Hale) 362, 368
Diffusing factors, substrates for hyaluronidase (Madinaveitia & Stacey) 413
Diiodotyrosine, conversion into thyroxine (Harington & Pitt Rivers) xxxiv
Diphenyl derivatives, antibacterial action of (Faulkner) 370
Diplococcin, anti-bacterial protein produced by milk streptococci (Oxford) 178
3:6-Disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine, as indicator in partition chromatography (Liddell & Rydon) 68
Drug-receptor, metabolic definition of (McIlwain) xxviii

Eel (*Anguilla anguilla* (L.)), chemistry of growth and food value of (McCance) 474
Egyptian scorpions, toxins of (Ahmed Hassan Mohammed) 284
Electrolytes, quenching effect of, on fluorescence of riboflavin and thiochrome (Ellinger & Holden) 147
Electrophoresis, of taka-diastase (McFarlane & Rimington) xxxiii
*Electrophoresis, preparative, with large Tiselius U-tube (Bunn & McFarlane) xxxv
Enzymes, *see also* under names of individual enzymes
Enzyme, formation by bacteria (Bray, Schlüchterer & Stacey) 154
Enzyme systems, action of clavatin on (Happold & Waters) xvii
Enzyme systems, inhibition of, by metabolic products of carcinogens (Elson & Hoch-Ligeti) x
Enzymes, combination of, with viruses (Kleczkowski) 160
Enzymes, inhibition of, by diamidines (Blaschkō & Duthie) xxv
Erythrocytes, *see also* Red blood corpuscles
Erythrocytes, fowl, effect of X-rays on respiration of (Frankenthal & Back) 351
Esterases, of skin (Thompson & Whittaker) 295
Ethanolamine, isolation of, from acid hydrolysates of gramicidin (Synge) xxxi
Evans blue, in plasma and serum, chromatography of (Morris) 203

Fat formation, in *Torulopsis lipofera* (Kleinzeller) 480
Fat, of human milk, component glycerides of (Hilditch & Meara) 437
Fats, milk, acids of (Hilditch & Jasperson) 443
Fatty acids, fractionation of, from solutions of crude bile salts, by adsorption on foam (Bader & Schütz) xxii
Fatty acids, in goat, ewe and mare milk (Hilditch & Jasperson) 443
Fatty acids, of human-milk fat (Hilditch & Meara) 29
Fermentation, by *Aeromonas hydrophila* (Stanier & Adams) 168
Ferrihaemoglobinaemia, in man and horse (Ramsay) 470
Fluorescence, of riboflavin and thiochrome, quenching effect of electrolytes on (Ellinger & Holden) 147
Fluorimetry, for estimation of 3:4-benzpyrene (Weil-Malherbe) 135, 141
Foam, fractionation of fatty and bile acids, from solution of crude bile salts, by adsorption on (Bader & Schütz) xxii
Food, estimation of vitamin B_6 in (Bottomley) v
Food, at R.A.F. messes, riboflavin content of (Macrae, Barton-Wright & Copping) 132
*Foods, nicotinic content of (Barton-Wright) xix
Formaldehyde, effect of, on tobacco mosaic virus (Kassanis & Kleczkowski) 20
Formaldehyde, reaction of, with histidine (Neuberger) 309
Fructose, effect of, on chain-length of liver glycogen (Bacon, Baldwin & Bell) 198
Fruits, carotene and lycopene in (Jacoby & Wokes) 279, i
Fruits, determination of ascorbic acid in (Harris & Mapson) xxv
Fruits and fruit products, vitamin P activity of (Bacharach & Coates) v
Fungi, wood-rotting, metabolic products of (Birkinshaw, Bracken & Findlay) 131

Galls, colouring matters of (Nierenstein & Swanton) 373
Gas production, by streptococci, as measure of growth (McIlwain) 97
Gelatin, methylation and acetylation of (Blackburn & Phillips) 171
Gestation, changes in skeleton of rat during (Warnock & Duckworth) 220
Glucose, effect of, on chain-length of liver glycogen (Bacon, Baldwin & Bell) 198
Glucuronic acid, determination of (Hanson, Mills & Williams) 274
Glycerides, of human-milk fat (Hilditch & Meara) 437
Glycerides, lower, formation of, during hydrolysis of triglyceride (Frazer & Sammons) xx
Glycogen, chain-length of (Bacon, Baldwin & Bell) 198
Gonadotrophin, in serum, purification of (Rimington & Rowlands) 54
Gramicidin, acid hydrolysates of, isolation of ethanolamine from (Synge) xxxi
Gramicidin, chemistry of (Synge) 285
Gram-staining, chemistry of (Henry) xxi
Growth factor, for *C. diphtheriae*, from liver (Chattaway, Happold & Sandford) 111
Growth factors, for *Lactobacillus casei* ϵ (Chattaway, Dolby & Happold) xviii
Growth, and *d*-lysine derivatives (Neuberger & Sanger) 125
Growth, of streptococcci, effects of pantothenate and pantoyltaurine on (McIlwain) 97
Growth, of streptococci, measurement of (McIlwain) 97
Gum tragacanth, chemistry of (James & Smith) xxi

*Haematins, formation of, from crystalline haemin and haemoglobin (King & Delory) vi
*Haemin, formation of haematins and haemochromogens from (King & Delory) vi
*Haemochromogens, formation of, from crystalline haemin and haemoglobin (King & Delory) vi
*Haemoglobin, formation of haematins and haemochromogens from (King & Delory) vi
*Haemoglobinimetry, accuracy of Sahli method (Bell & McNaught) xv
*Haemolymphatic changes, in lymph-nodes of rat, after administration of 1:2:5:6-dibenzanthracene (Lasnitzki & Woodhouse) xxii
Hair, dicarboxylic and basic amino-acids of (Beveridge & Lucas) 88
Hair, human, serine in (Beveridge & Lucas) 411
Hair, isolation of proline from (Beveridge & Lucas) 95
Hair keratin, analysis of (Beveridge & Lucas) 88, 95, 411
Heart, destruction of acetic acid by (Barcroft, McAnally & Phillipson) iv
Hemicellulose, extraction of (Preece) 402
Histaminase, in normal and toxaemic pregnancy (Kapeller-Adler) 270
Histidine, excretion of, effect of vitamin B_1 on (Dawson) xvii
Histidine, reaction of, with formaldehyde (Neuberger) 309
Histone, retardation of growth of mouse carcinoma by (Stedman, Stedman & Pettigrew) xxxi
Horse serum, purification of pseudo-cholinesterase from (Strelitz) 86
Human milk, fatty acids of (Hilditch & Meara) 29
Hyaluronic acid, action of reducing agents on (Hale) 362
Hyaluronidase, activity of (Hale) 368
Hyaluronidase, influence of environmental conditions on activity of (Hale) 368
Hyaluronidase, production of, by micro-organisms (Rogers) xxxiii
Hyaluronidase, substrates for (Madinaveitia & Stacey) 413

Indicator, for partition chromatography (Liddell & Rydon) 68
Infants, β-carotene in serum of (Hoch) 304
Iron, micro-determination of (Ramsay) 467

Keratin of hair, analysis of (Beveridge & Lucas) 88, 95, 411
Ketonic acids, derived from cholic acid (Haslewood) 108

Lactation, changes in skeleton of rat during (Warnock & Duckworth) 220

Lactobacillus arabinosus, microbiological assay of nicotinic acid with (Barton-Wright) 314
Lactobacillus casei ϵ, growth factors for (Chattaway, Dolby & Happold) xviii
Lactobacillus helveticus, growth factors for, from liver (Barton-Wright, Emery & Robinson) xviii
Leaves, plant, extraction of proteins from (Lugg & Weller) 408
Lipoid pigments, in palm oils (Hunter, Scott & Williams) 209
Lipoid pigments, in palm oils (Hunter & Scott) 211
Liver arginase, in urea synthesis (Bach, Crook & Williamson) 325
Liver cytoplasm, effect of dietary protein on (Kosterlitz) xiv
Liver glycogen, chain-length of, effect of glucose, fructose and sucrose on (Bacon, Baldwin & Bell) 198
Liver, growth factor from, for *C. diphtheriae* (Chattaway, Happold & Sandford) 111
Liver, growth factors from, for *Lactobacillus helveticus* and *Streptococcus lactis* R. (Barton-Wright, Emery & Robinson) xviii
Liver, isolation of ribonucleic acid from (Davidson & Waymouth) xiii
Liver, nucleic acid content of (Davidson & Waymouth) 379
Liver, nucleotide content of (Davidson & Waymouth) 379
Liver, nucleotide and polynucleotide content of (Davidson & Waymouth) xiii
Liver, ribonucleic acid from (Davidson & Waymouth) 375
Liver slices, arginase activity of (Bach) 156
Liver storage test, for vitamin A (Guggenheim & Koch) 256
Lungs, of South Wales miners, mineral content of (King & Nagelschmidt) vi
Lycopene, in palm oils (Hunter, Scott & Williams) 209
Lycopene, in palm oils (Hunter & Scott) 211
Lycopene, in rose hips and other fruits (Jacoby & Wokes) 279, i
*Lymph-nodes, of rat, haemolymphatic changes in, after administration of 1:2:5:6-dibenzanthracene (Lasnitzki & Woodhouse) xxii
l(+)-Lysine decarboxylase, from bacteria (Gale & Epps) 232
d-Lysine derivatives, and growth (Neuberger & Sanger) 125
Lysine, metabolism of (Neuberger & Sanger) 119
*Lysine requirement, of adult rat (Neuberger & Webster) xxxv

Manna, isolation of trehalose from (Leibowitz) 205
Mastication, effect of, on ascorbic acid content of raw vegetables (Jenkins) xix
Mattick, E. C. V., obituary notice of (Kay) 1
Mercuric chloride, effect of, on tobacco mosaic virus (Kassanis & Kleczkowski) 20
Metabolism, of acetoacetic acid (Krebs & Eggleston) xxix
Metabolism, of azobenzene (Elson & Warren) 217
Metabolism, of lysine (Neuberger & Sanger) 119
Metabolism, of moulds (Mann) 339, 345
Metabolism, oxidative, of *Ascaris suis* (Laser) 333
Metabolism, of *Penicillium sclerotiorum* van Beyma, effect of nitrate on (Reilly, Long & Curtin) 37
Metabolism, of phosphorus, in moulds (Mann) 339
Metabolism, of steroids (Haslewood) 108
Metabolism, of streptococci, effects of pantothenate and pantoyltaurine on (McIlwain) 97
Metabolism, of 2:4:6-trinitrotoluene (Channon, Mills & Williams) 70
Metaphosphate, from *Aspergillus niger* (Mann) 345
Methaemoglobinaemia, case of (King, Gilchrist & White) viii
Methaemoglobinaemia, in man and horse (Ramsay) 470
Methionine content, of plant leaf proteins (Lugg & Weller) 408
Methylation, of thiol groups of bisulphited wools (Blackburn, Consden & Phillips) 25
Methylation, of wool, silk fibroin, collagen and gelatin (Blackburn & Phillips) 171
**N*-Methyl-chloro-nicotinamide, in urine, estimation of (Coulson, Ellinger & Holden) v
ϵ-Methyl-*dl*-lysine, and growth (Neuberger & Sanger) 125
Mice, estimation of 3:4-benzpyrene in (Weil-Malherbe) 135
Micro-organisms, absence of displacement of pantothenate and *p*-aminobenzoate from, by pantoyltaurine and sulphanilamide (McIlwain) viii
Micro-organisms, production of hyaluronidase by (Rogers) xxxiii
*Milk, apparatus for measuring abnormality of (Davis & Ward) xi
Milk fat, fatty acids of (Hilditch & Meara) 29
Milk fats, of goat, ewe and mare, fatty acids of (Hilditch & Jasperson) 443
Milk, human, glycerides of (Hilditch & Meara) 437
Milk, rapid examination of, for chemical and bacteriological quality (Davis & Ward) ix
Milk streptococci, bacteriostatic protein produced by (Oxford) 178
Mineral content, of lungs of South Wales miners (King & Nagelschmidt) vi
Molybdate, reaction of anthocyanins with (Blaschko) vi
Monoglycerides, formation of, during hydrolysis of triglyceride (Frazer & Sammons) xx
Mono-iodotyrosine (Harington & Pitt Rivers) 320
Moulds, metabolism of (Mann) 339, 345
Moulds, phosphorus metabolism in (Mann) 339, 345
Moulds, pyrophosphate and metaphosphate in (Mann 345
Mouse carcinoma 2146, retardation of growth of, by histone and protamine (Stedman, Stedman & Pettigrew) xxxi
*Mucoprotein, crystalline, with high choline esterase activity (Bader, Schütz & Stacey) xx

Naphthoresorcinol reaction, for determination of glucuronic acid (Hanson, Mills & Williams) 274
α-Naphthylamine, 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-, as indicator in partition chromatography (Liddell & Rydon) 68
Nicotinamide methochloride, urinary elimination of (Ellinger & Coulson) 265
Nicotinamide methochloride, in urine, estimation of (Coulson, Ellinger & Holden) 150
*Nicotinamide, *N*-methyl-chloro-, in urine, estimation of (Coulson, Ellinger & Holden) v
Nicotinic acid, in cereals, microbiological assay of (Barton-Wright) 314
*Nicotinic acid content, of cereals and other foods (Barton-Wright) xix
*Nicotinic acid, microbiological assay of (Barton-Wright) xix
Nitrate, effect of, on metabolism of *Penicillium sclerotiorum* van Beyma (Reilly, Long & Curtin) 37
Nuclei, of salmon cells, basic proteins of (Stedman & Stedman) xxvi
Nucleic acids, in embryonic and adult tissue (Davidson & Waymouth) 39
Nucleic acids, in liver (Davidson & Waymouth) 379
Nucleic acids, in tissues (Davidson & Waymouth) 375, 379
Nucleotide, in liver (Davidson & Waymouth) 379, xiii
Nucleotides, in embryonic and adult tissue (Davidson & Waymouth) 39
Nutrition, of boys (Cook, Davidson, Keay & McIntosh) xiii
*Nutrition, of pregnant women (Young, Wood & King) vi

Oats, aneurin in, determination of (Holman) 388
Obituary notice, of E. C. V. Mattick (Kay) 1
Obituary notice, of F. L. Pyman (Anderson) 283
Oils, palm, carotenoids in (Hunter, Scott & Williams) 209
Oils, palm, carotenoids in (Hunter & Scott) 211
Ovarian cyst, pseudo-mucinous, fluids of, isolation of blood group A substance from (King & Morgan) x
Oxidative metabolism, of *Ascaris suis* (Laser) 333

Palm, oils, carotenoids in (Hunter, Scott & Williams) 209
Palm, oils, carotenoids in (Hunter & Scott) 211
Pancreatic lipolysis, formation of lower glycerides during (Frazer & Sammons) xx
Pantothenate, action of, on streptococci (McIlwain & Hughes) 187
Pantothenate, effects of, on metabolism and growth of streptococci (McIlwain) 97
Pantothenate, non-displacement of, from micro-organisms, by pantoyltaurine (McIlwain) viii
Pantothenic acid, antagonists of, antibacterial activity of (Madinaveitia, Martin, Rose & Swain) xxvii
Pantoyltaurine, action of, on streptococci (McIlwain & Hughes) 187
Pantoyltaurine, effects of, on metabolism and growth of streptococci (McIlwain) 97
Pantoyltaurine, non-displacement of pantothenate from micro-organisms by (McIlwain) viii
Paper, filter, in chromatography of proteins (Consden, Gordon & Martin) 224
Partition chromatography, 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine as indicator in (Liddell & Rydon) 68
Partition chromatography, of proteins, with paper (Consden, Gordon & Martin) 224
Partition chromatography, with silica gel, of acetamino-acids (Gordon, Martin & Synge) 65
Partition chromatography, with starch, of gramicidin hydrolysate (Synge) 285
Patulin, enzymic hydrolysis of (Krebs) xxix
Penicillin, assay of (Heatley) 61
*Penicillin, assay of (White) xxxv
Penicillin, preparation of (Clayton, Hems, Robinson, Andrews & Hunwicke) 452
Penicillium sclerotiorum van Beyma, metabolism of, influence of sodium nitrate concentration on (Reilly, Long & Curtin) 37
Pepsin, combination of potato virus *X* and tobacco mosaic virus with (Kleczkowski) 160
Pepsin, crystalline, action of, on cysteine-(cystine-) tyrosine peptides (Harington & Pitt Rivers) 417
Peptides, cysteine-(cystine-) tyrosine, action of crystalline pepsin on (Harington & Pitt Rivers) 417
Peptides, cysteine-(cystine-) tyrosine, synthesis of (Harington & Pitt Rivers) 417
9-Phenyl-5:6-benzo-*iso*-alloxazine, induction of coat coloration in albino rat by (Haddow, Elson, Rudall, Timmis & Roe) xxxiv, xxxv
Phosphatase, 'activation' of (Delory & King) 50
Phosphatase, plasma acid, in carcinoma of prostate, effect of stilboestrol on (Watkinson, Delory, King & Haddow) iv
Phosphatases, acid, differentiation of, in sera (Herbert) xxiii
Phosphatases, in cereals (Booth) 355
Phosphatases, in wheat (Booth) 355
Phosphomonoesterase, in wheat, assay and characterization of (Booth) 355
Phosphorus metabolism, in moulds (Mann) 339, 345
Photoelectric absorptiometer, determination of glucuronic acid with (Hanson, Mills & Williams) 274
Photometer, for field and emergency use (King & Delory) vii
Pigmentation, of coat of albino rat, induced by 9-phenyl-5:6-benzo-*iso*-alloxazine (Haddow, Elson, Rudall, Timmis & Roe) xxxiv, xxxv
Pigments, in palm oils (Hunter, Scott & Williams) 209
Pigments, in palm oils (Hunter & Scott) 211
Plant extracts, reduction of dehydroascorbic acid in (Crook & Morgan) 10
Plant leaf proteins, amino-acid contents of (Lugg & Weller 408
Plant leaves, extraction of proteins from (Lugg & Weller 408
Plants, determination of cellulose in (Shrikhande) 207
Plasma acid phosphatase, in carcinoma of prostate, effect of stilboestrol on (Watkinson, Delory, King & Haddow) iv
Plasma ascorbic acid level and capillary resistance, correlation between (Scarborough & Gilchrist) i
Plasma, chromatography of Evans blue in (Morris) 203
Plasma, determination of specific gravity of (Plimmer) vii
Plasma proteins, in boys (Cook, Keay & McIntosh) xiv
*Plasma volume, in adrenal insufficiency (Cleghorn, Ferguson & Hanna) vi
Polynucleotide, in liver (Davidson & Waymouth) xiii
Polysaccharide production, from sucrose (Hestrin & Avineri-Shapiro) 2
Polysaccharide, synthesis by *Rhizobium radicicolum* (Bray, Schlüchterer & Stacey) 154
Polysaccharides, action of reducing agents on (Hale) 362
Potato virus *X*, combination of, with pepsin and trypsin (Kleczkowski) 160
Pregnancy, normal and toxaemic, histaminase in (Kapeller-Adler) 270
*Pregnant women, nutrition of (Young, Wood & King) vi
Proactinomyces spp., oxidation of cholesterol by (Turfitt) 492
Proline, from human hair, isolation of (Beveridge & Lucas) 95
Propionic acid, absorption of (Barcroft, McAnally & Phillipson) iii
Propionic acid, production of, in alimentary canal (Barcroft, McAnally & Phillipson) ii
Prostate, carcinoma of, effect of stilboestrol on plasma acid phosphatase in (Watkinson, Delory, King & Haddow) iv
Prostatic phosphatase, in sera (Herbert) xxiii
Protamine, retardation of growth of mouse carcinoma by (Stedman, Stedman & Pettigrew) xxxi
Protein, bacteriostatic, produced by milk streptococci (Oxford) 178
Protein derivatives, effect of, on absorption of calcium (Hall & Lehmann) 117
Protein, in diet, effect of, on liver cytoplasm (Kosterlitz) xiv
Protein synthesis, in bovine rumen (Smith & Baker) 496
Proteins, basic, of salmon cell-nuclei (Stedman & Stedman) xxvi
Proteins, extraction of, from plant leaves (Lugg & Weller) 408
Proteins, partition chromatography of, with paper (Consden, Gordon & Martin) 224
Proteins, plant leaf, amino-acid contents of (Lugg & Weller) 408
Proteins, of plasma, in boys (Cook, Keay & McIntosh) xiv
Pseudo-cholinesterase, from horse serum, purification of (Strelitz) 86
Pseudo-mucinous ovarian cyst, fluids of, isolation of blood group A substance from (King & Morgan) x
Pyman, F. L., obituary notice of (Anderson) 283
Pyrophosphatase, from yeast (Bailey & Webb) 394
Pyrophosphate, from *Aspergillus niger* (Mann) 345
Pyruvic acid, in blood, stabilization and estimation of (Long) 447, xxvii

'*Q*' notation, use of (Bach) 156

*Rat, lysine requirement of (Neuberger & Webster) xxxv
Rat, skeleton of, changes in, during gestation and lactation (Warnock & Duckworth) 220
Red blood corpuscles, *see also* Erythrocytes
Red blood corpuscles, fowl, effect of X-rays on respiration of (Frankenthal & Back) 351
Reducing agents, action of, on hyaluronic acid polysaccharides (Hale) 362
Reduction, of dehydroascorbic acid (Crook & Morgan) 10
Respiration, of fowl erythrocytes, effect of X-rays on (Frankenthal & Back) 351
Rhizobium radicicolum, formation of polysaccharide by (Bray, Schlüchterer & Stacey) 154
Riboflavin, *see also* Vitamin B_2
Riboflavin, antagonism between antimalarial drugs and (Madinaveitia) xxvii
Riboflavin, in canteen meals (Kerly) 423
Riboflavin, in food at R.A.F. messes (Macrae, Barton-Wright & Copping) 132
Riboflavin, quenching effect of electrolytes on fluorescence of (Ellinger & Holden) 147
Ribonucleic acid, from liver (Davidson & Waymouth) 375
Ribonucleic acid, from liver, isolation and properties of (Davidson & Waymouth) xiii
Ribonucleic acids, in embryonic and adult tissue (Davidson & Waymouth) 39
Rose hips, carotene and lycopene in (Jacoby & Wokes) 279, i
Royal Air Force messes, riboflavin content of food at (Macrae, Barton-Wright & Copping) 132
Rumen, bovine, protein synthesis and microbial activities in (Smith & Baker) 496
Rumen, bovine, utilization of urea in (Smith & Baker) 496

*Sahli haemoglobinimetry, accuracy of (Bell & McNaught) xv
Scorpions, Egyptian, toxins of (Ahmed Hassan Mohammed) 284
Scutellum, of wheat germ, chemistry of (Hinton) 214
Sera, differentiation of acid phosphatases in (Herbert) xxiii
Serine, in human hair (Beveridge & Lucas) 411
Serum calcium, estimation of (de Loureiro & Janz) 16
Serum, chromatography of Evans blue in (Morris) 203
Serum, determination of specific gravity of (Plimmer) vii
Serum gonadotrophin, purification of (Rimington & Rowlands) 54
Serum, of infants, β-carotene in (Hoch) 304
*Serum proteins, in adrenal insufficiency (Cleghorn, Ferguson & Hanna) vi
Serum, pseudo-cholinesterase from (Strelitz) 86
Serum, thiocyanate in, determination of (Bowler) 385
Silica gel, for chromatography of acetamino-acids (Gordon, Martin & Synge) 65
Silk fibroin, methylation and acetylation of (Blackburn & Phillips) 171
Skeleton, of rat, changes in, during gestation and lactation (Warnock & Duckworth) 220
Skin, esterases of (Thompson & Whittaker) 295
Sodium nitrate, effect of, on metabolism of *Penicillium sclerotiorum* van Beyma (Reilly, Long & Curtin) 37
Solid-liquid interface, adsorption on, temperature factor in (Bader & Schütz) xx
South Wales miners, mineral content of lungs of (King & Nagelschmidt) vi
Specific gravity, of blood, plasma and serum, measurement of (Plimmer) vii
Starch, chromatography of gramicidin hydrolysate with (Synge) 285
Starch, as medium for chromatography (Elsden & Synge) ix
Steroids, metabolism of (Haslewood) 108
Steroids, microbiological degradation of (Turfitt) 492
Stilboestrol, effect of, on plasma acid phosphatase in carcinoma of prostate (Watkinson, Delory, King & Haddow) iv
Streptococci, actions of pantothenate and pantoyltaurine on (McIlwain & Hughes) 187
Streptococci, effects of pantothenate and pantoyltaurine on metabolism and growth of (McIlwain) 97
Streptococci, measurement of growth of (McIlwain) 97
Streptococci, of milk, bacteriostatic protein produced by (Oxford) 178
Streptococcus faecalis, *l*(−)-tyrosine decarboxylase from (Epps) 242
Streptococcus lactis R., growth factors for, from liver (Barton-Wright, Emery & Robinson) xviii
Succinoxidase, inhibition of, by metabolic products of carcinogens (Elson & Hoch-Ligeti) x
Sucrose, effect of, on chain-length of liver glycogen (Bacon, Baldwin & Bell) 198
Sucrose, polysaccharide production from (Hestrin & Avineri-Shapiro) 2
Sulphamezathine, metabolism of (Sykes) xxix
Sulphanilamide, non-displacement of *p*-aminobenzoate from micro-organisms by (McIlwain) viii
Sulphanilamides, estimation of (Rose & Bevan) 116
N-β-Sulphatoethyl-*m*-toluidine, for estimation of sulphanilamides (Rose & Bevan) 116
Sulphites, action of, on disulphide linkages of wool (Blackburn, Consden & Phillips) 25

Taka-diastase, *N*-acetylglucosaminidase in (Rimington) xxxii
Taka-diastase, electrophoresis of (McFarlane & Rimington) xxxiii
Tannins, of cacao bean, changes in, during autolysis (Humphries) 182
Temperature factor, in adsorption on solid-liquid interface (Bader & Schütz) xx
Tetrathionase, of bacteria, adaptation without cell growth (Knox & Pollock) 299
Thiochrome, oxidation of aneurin to (Holman) 388
Thiochrome, quenching effect of electrolytes on fluorescence of (Ellinger & Holden) 147
Thiocyanate, in blood serum, determination of (Bowler) 385
Thiol groups, of bisulphited wool, methylation of (Blackburn, Consden & Phillips) 25
*Thio-uracil, estimation of (Anderson) xv
Thyroxine, conversion of diiodotyrosine into (Harington & Pitt Rivers) xxxiv
Tissue slices, '*Q*' notation for expressing metabolic activities in (Bach) 156
Tissues, animal and plant, codecarboxylase in (Gale & Epps) 250
Tissues, embryonic and adult, ribonucleic acids and nucleotides in (Davidson & Waymouth) 39
Tissues, nucleic acids of (Davidson & Waymouth) 375, 379
Tobacco mosaic virus, combination of, with pepsin and trypsin (Kleczkowski) 160
Tobacco mosaic virus, effect of formaldehyde and mercuric chloride on (Kassanis & Kleczkowski) 20
Tobacco mosaic virus, inactivation of (Kassanis & Kleczkowski) 20
Torulopsis lipofera, fat formation in (Kleinzeller) 480
Toxaemia, of pregnancy, histaminase in (Kapeller-Adler) 270
Toxins, of Egyptian scorpions (Ahmed Hassan Mohammed) 284
Tragacanth, gum, chemistry of (James & Smith) xxi
Trametes suaveolens (Linn.) Fr., metabolic products of (Birkinshaw, Bracken & Findlay) 131
Trehalose, isolation of, from desert manna (Leibowitz) 205
Triglyceride, hydrolysis of, formation of lower glycerides during (Frazer & Sammons) xx

2:4:6-Trinitrotoluene, metabolism of (Channon, Mills & Williams) 70
Trypsin, combination of potato virus *X* and tobacco mosaic virus with (Kleczkowski) 160
Tyrosine content, of plant leaf proteins (Lugg & Weller) 408
l(−)-Tyrosine decarboxylase, from *Streptococcus faecalis* (Epps) 242
Tissues, chemistry of (Bray, Gregory & Stacey) 142
Tryptophan content, of plant leaf proteins (Lugg & Weller) 408

Urea synthesis, and arginase (Bach, Crook & Williamson) 325
Urea, utilization of, by ruminants (Smith & Baker) 496
Urease, inhibition of, by metabolic products of carcinogens (Elson & Hoch-Ligeti) x
Uric acid, estimation of (Fearon) 399
Urinary excretion of histidine, effect of vitamin B_1 on (Dawson) xvii
Urine, androstenetriol in (Marrian & Butler) 322
Urine, elimination of nicotinamide methochloride in (Ellinger & Coulson) 265
*Urine, estimation of *N*-methyl-chloro-nicotinamide in (Coulson, Ellinger & Holden) v
Urine, estimation of nicotinamide methochloride in (Coulson, Ellinger & Holden) 150
Urine, separation of arsenite from arsenate in (Crawford & Storey) 195

Vegetables, determination of ascorbic acid in (Harris & Mapson) xxv
Vegetables, raw, ascorbic acid content of, effect of mastication on (Jenkins) xix
Virus, tobacco mosaic, combination of, with pepsin and trypsin (Kleczkowski) 160
Virus, tobacco mosaic, effect of formaldehyde and mercuric chloride on (Kassanis & Kleczkowski) 20
Virus, tobacco mosaic, inactivation of (Kassanis & Kleczkowski) 20
Virus *X*, of potato, combination of, with pepsin and trypsin (Kleczkowski) 160
Viruses, combination of enzymes with (Kleczkowski) 160
Vitamin A, assessment of, by liver storage test (Guggenheim & Koch) 256
Vitamin A, 'cyclization' of (Hawkins & Hunter) 34
Vitamin A, utilization of, effect of vitamin E on (Guggenheim) 260
Vitamin B_1, *see also* Aneurin
Vitamin B_1, effect of, on histidine excretion (Dawson) xvii
Vitamin B_6, *see also* Riboflavin
Vitamin B_6, in food, estimation of (Bottomley) v
Vitamin C, *see also* Ascorbic acid
Vitamin C, synthesis of, in stored apples (West & Zilva) 105
Vitamin E, chemical estimation of (Tošić & Moore) xxvi
Vitamin E, effect of, on utilization of carotene and vitamin A (Guggenheim) 260
Vitamin P, in fruits and fruit products (Bacharach & Coates) v

Wheat germ, chemistry of (Hinton) 214
Wheat, phosphatase in (Booth) 355
Wheat, phosphomonoesterase in, assay and characterization of (Booth) 355
*Women, pregnant, nutrition of (Young, Wood & King) vi
Wood-rotting fungi, metabolic products of (Birkinshaw, Bracken & Findlay) 131
Wool, action of sulphites on disulphide linkages of (Blackburn, Consden & Phillips) 25
Wool, bisulphited, methylation of thiol groups of (Blackburn, Consden & Phillips) 25
Wool, methylation and acetylation of (Blackburn & Phillips) 171

X-rays, effect of, on respiration of fowl erythrocytes (Frankenthal & Back) 351

Yeast, pyrophosphatase from (Bailey & Webb) 394
Yeasts, preparation of codecarboxylase from (Gale & Epps) 250

CAMBRIDGE: PRINTED BY WALTER LEWIS, M.A., AT THE UNIVERSITY PRESS